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14. ABSTRACT

The increasing frequency of fungal infections and broadening spectrum of organisms that cause these infections has placed a growing burden on clinical microbiologists. To address this problem, the goal of this proposal is to develop a universal sequence-based identification system that only requires basic laboratory skills to utilize. This goal will be accomplished by 1) Creating an internet-accessible sequence database for fungal identification, 2) Developing standardized protocols for these analyses, 3) generating the sequences for the database, and 4) Confirming the utility of the database by comparison to clinical testing protocols. The focus of the work during this funding period mainly centered around Task 3, which was directed towards obtaining Type culture sequences and depositing these sequences into our database. These sequences form the centerpiece of this proposal and represent the target DNA sequences of the original fungal strain from which the species name was first described. Therefore, just as the "Type culture" is the original strain from which all the characteristics of the species are defined, the "Type sequence" is the defining sequence by which a molecular identification of the particular species can be made. In addition to obtaining Type sequences and depositing them into the database (subtask 1), the second subtask consisted of obtaining additional sequences from clinical isolates that we have at our institution and depositing these sequences into our database as well. The findings during this phase of the study are that we have been able to successfully recover sequences from Type cultures that were obtained from culture repositories and add them to our database. We have also added sequences from clinical isolates from our institution's culture collection, and also have developed methodology to identify and add clinical isolate sequences as they come in from patients, if we deem them interesting/important or novel. These findings have built upon our earlier task completion, which included development of the database and standardization of the methodology. We now have a working system that will enable completing the last task of the study.

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INTRODUCTION: This proposal focuses on the development of a system for the molecular identification of human fungal pathogens using a biocurated database of DNA sequences. While common fungi can be easily identified by clinical microbiologists, identification of non-routine fungi from clinical specimens cannot be reliably done without specific training in mycology. Unfortunately, individuals with this training are in short supply in both civilian and military hospitals. The objective of this study is to enable laboratory technicians to make proper identifications without experience in mycology using standardized techniques developed in this proposal to generate a DNA sequence, which can then be used to search an internet-accessible database developed by our laboratory. The output from searches of this database will be reconfigured from the standard BLAST algorithm search output currently used by Genbank (the largest sequence repository in the world) to yield an accurate identification that utilizes proper and consistent fungal nomenclature. This strategy will allow technicians to provide an appropriate identification, enabling clinicians to more efficiently select the proper treatment course. The significance of our study will be to enable any clinical laboratory, regardless of mycological expertise, to identify any human fungal pathogen faster and more accurately than is presently possible, using a single assay.

BODY: This reporting period is the third of this award and describes work in progress on the third of the four tasks (tasks 1 and 2 were completed by the last reporting period). Presently all tasks are on schedule and no changes to the original Statement of Work have been made. No major problems have been encountered, and because our first year was ahead of schedule, we have expanded the work on the database through more frequent meetings with our programming group. Since tasks one and two are complete, they won't be elaborated on in this report, however, they consisted of: Task 1: Creation of an internet-accessible, sequence database for the molecular identification of all known human fungal pathogens. (Months 1-12), and Task 2: Development of standardized protocols for PCR and sequencing template preparation. (Months 6-18). The third task is the subject of this report and consists of Aim 3. Generation of type sequences. (Months 6-36).

The main aspect of our database consists of an internet portal that allows access to the database through the BLASTn search algorithm. The database is accessed through a UTHSCSA site (http://pfris.uthscsa.edu/), which takes users to the face page (Fig 1). This page has multiple links that direct users to information about the site, how to do searches, how to prepare sequences, and background information about the database. Other links are included to provide information about medical mycology in general, and particular species of fungi in particular. Presently we have almost six hundred cultures in our collection. We have obtained about five hundred sequences and deposited these sequences, after careful quality control to insure that they are correct and accurate, into the database. In addition to reference sequences (Type cultures, genomic sequences, culture collection isolates) we also identify and incorporate isolates that may be unusual or not reported as human pathogens. In particular, we are interested in fungi that could be potential zoonotic agents, and have used molecular sequencing to identify a number of these isolates from a wide range of animals (1,3,8,10,13,15,16,17). Given the presence of the United States military in Iraq and Afghanistan, we have also been interested in isolates that have origins in these regions and have published one report on a case involving an isolate from the

middle east (5). Other studies have included the molecular identification of isolates that infect the immunosuppressed (1,2,4,6), which are of interest because these are rare infections that can be impossible to identify and difficult to treat, and support for a genome sequencing project centered at MIT that used one of our strains (7). Finally we have also collaborated on fungal infections in the chronically ill (9) and are in discussions to conduct a broad-based study of fungal infections in patients with cystic fibrosis in which we will do all of the identifications using our database. Finally, in addition to the searchable function of our database, we have also intended this proposal to serve as a vehicle for obtaining and storing high quality sequence information that could be used in other platforms (11,12). These studies rely on the data we have added to the database for other purposes, such as developing new diagnostic reagents using the sequence data to design new probes.

Fig. 1. The web portal for the PFRIS database. The site is hosted at UTHSCSA. Search access is initiated using the "Proceed to PFRIS" button, which pulls up the BLAST window. The search query sequence is then pasted into the window and the search is initiated.

In order to capture and continually add new potentially pathogenic fungal sequences into our database, we have established a link with the UTHSCSA Dept. of Pathology, Fungus Testing Laboratory. This laboratory regularly obtains isolates from all over the world that clinical microbiologists have not been able to identify. In order to follow these isolates and identify sequences of interest, we have recently begun building a second database that is an internal database that runs parallel to our main, PFRIS database. This database is the Fungus Testing Laboratory Clinical Isolate Database, which we will use as a tracking database. Any fungus that comes into the laboratory that we feel is of interest, we can, by a simple process, import this sequence and all the information attached to it, into our own database. This strategy will contribute greatly to our original goal of making the PFRIS database a dynamic database that is

continually updated with new sequence. The approach will greatly strengthen our sequence redundancy by allowing us to easily add multiple sequences for each species of fungus.

Finally, now that our main database is operation (the PFRIS database), we continually quality control test the database, and look for ways to improve its power or functionality. We intend, in future studies, to expand the functionality of this database by allowing users to search the database for information that they may find useful. To more efficiently accomplish this task, data in the database needs to be ordered and classified in more detail. Therefore, during this most recent period we have added functionality by adding additional fields to the main database. These fields are based on the National Center for Biotechnology, Genbank database, and consist of fields that allow a more detailed taxonomic classification of data. The Genbank database has a Taxon number that is unique for every species of organism. We have added a field for these numbers, based on the Genbank database, so that a single number can be searched if someone wants to find all of the records in our database that relate to one species of fungus. We then extended what the Genbank database does and added separate fields that will hold phylogenetic information. These fields consist of entries for Phylum, Class, Order, and Family. Searching the database using these fields will allow users to group records by different taxonomic criteria, and then investigate the sequences depending on their search parameters. We have found these modifications to the database to be relatively easy to perform and will continue to make additional improvements in the coming year.

KEY RESEARCH ACCOMPLISHMENTS: Most of the key research accomplishments have centered around publications, however, we have also established collaborations with military hospitals, made presentations, and continued to expand the programming aspect of this study. A brief list of key accomplishments is as follows:

A. The first two tasks of this proposal were technical in nature and have been completed and applied to task three. We now have standardized methods for obtaining sequences from isolates, and a functional database to deposit the sequences in.

B. The database, developed in task 1, was debugged during this last reporting period, and is now fully operational and used for identifications. Sequences are being continuously added (and will be throughout this study), and searches are being conducted. Importantly, we continue to add functionality to the database through the addition of new fields.

C. Because of the relatively problem-free creation of the main database, we have also started, and have almost completed, a second parallel database that will allow us to monitor clinical isolates that come into the Department of Pathology for identification. Important/novel/interesting isolates and their sequences can then be easily imported into our main database with a few key-strokes.

D. We have assumed a role as collaborator on a new grant with investigators at Brooks Army Medical Center that will work with fungi isolated from military burn patients. A second collaboration is planned with investigators at the, Walter Reed Army Medical Center, Washington, DC, Uniformed Services University, Bethesda, MD, for fungal identification using

our database.

E. We have published a recommendation on molecular sequencing for difficult to identify fungi (14), in conjunction with the molecular diagnostics group, that we were asked to join during the last period, which represents official positions of the International Society for Human and Animal Mycology. The paper proposes official strategies for investigators using molecular identification as a diagnostic tool.

REPORTABLE OUTCOMES:

I. The two previous progress reports listed one publication per year (2006, 2007). During this third year period, we have published, in press, or submitted an additional 18 reports of our work. The reports during this period are broken down as 3 published, 4 in press, and 10 submitted. In total, 19 manuscripts have been submitted, published, or are accepted in the three years that this proposal has been funded.

II. Five additional abstracts have been presented at local, regional, or national meetings, for a total of 10 abstracts during the three years that this proposal has been funded.

III. Three invited seminars were presented:

Nov 2008 "Strain-Dependent Variation of 18S rDNA Copy Number in *Aspergillus fumigatus*" Invasive Aspergillus and Aspergillosis workshop. National Institutes of Health, Bethesda, MD.

Dec 2008 "What's the Fungus!!? -- Can Molecular Identification Find a Place in the Clinical Mycology Laboratory?" San Antonio Center for Medical Mycology, San Antonio, TX

Jan 2009 " The Changing Landscape of Fungal Identification: Morphology and Molecules" Department of Pathology, The University of Texas Health Science Center at San Antonio".

IV. Funding

New: "Laser Microdissection (LMD) with DNA PCR Amplification and Sequencing: A Novel Method for Determining the Etiology of Fungal Burn Wound Infection" PI, Davignon, Laurie, Major, MD, Maj, USAF, MC, Assistant Chief, Infectious Disease Service, San Antonio Military Medical Center, Fort Sam Houston, TX 78234-6200. Sponsor: Brooks Army Medical Center-Fort Sam Houston. Role: Consultant.

Continuing (from 1st reporting period.) "Detection and significance of antifungal resistance in oropharyngeal candidiasis". PI, Tom Patterson, MD, Chief, Infectious Disease and Professor, Dept. of Medicine, University of Texas Health Science Center at San Antonio. Sponsor: NIH, National Institute of Dental and Craniofacial Research (NIDCR). Awarded 07/06 and runs until 06/11. Role: Co-I.

Funding Applied For "A Biocurated Sequence Database for Fungal Identification." Sponsor: NIH, National Institutes of Allergy and Infectious Diseases. Role: Principal Investigator

V. Publications:

Sutton, D.A., **Wickes, B.L.**, Romanelli, A.M., Rinaldi, M.G., Thompson, E.H., Fothergill, A.W., Dishop, M.K., Elidemir, O., Mallory, G.B., Moonnamakal, S.P., Adesina, A.M., and M.G. Schecter. 2009. Fatal Central Nervous System *Aspergillus granulosus* in a Lung Transplant Recipient. Submitted.

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Readinger, A., LaSala, P.R., McGinnis, M.R., **Wickes, B.L**., Sutton, D.A., and M.G. Wilkerson. 2009. Hyalohyphomycosis caused by the coelomycete *Microsphaeropsis arundinis* in a cadaveric renal transplant patient. Submitted

Taj-Aldeen, S.J., Almaslamani, M., Alkhal, A, Al Bozom, I., El Shafie, S, Al-Ansari, N., Romanelli, A.M., **Wickes, B.L.,** Fothergill, A., and D.A. Sutton. 2009. Cerebral phaeohyphomycosis due to *Rhinocladiella mackenziei* (formerly *Rhamichloridium mackenziei*): a rare cause of fungal brain abscess. Submitted

Srinivasan, A., **Wickes,B.L**., Rubnitz, J.M., Sutton, D.A., Romanelli, A.M., Shenep, J.L. and R.T. Hayden. 2009. *Macrophomina Phaseolina* presenting as a skin infection in a child with acute leukemia. Submitted

Ma, L.J., A.S. Ibrahim, C. Skory, M.G. Grabherr, G. Burger, F. Lang, A. Abe, M. Butler, S. Calvo, L.M. Corrochano, M. Elias, R. Engels, J. Fu, W. Hansberg, A. Idnurm, J-M. Kim, C.D. Kodira, M.J. Koehrsen, B. Liu, D. Miranda-Saavedra, J. Rodriguez-Romero, S. O'Leary, L. Ortiz-Castellanos, R. Poulter, J. Ruiz-Herrera, Y. Shen, T. Sone, Q. Zeng, J. Galagan, B.W. Birren, C.A. Cuomo, and **B.L. Wickes**. 2009. Genome analysis of the opportunistic pathogen, *Rhizopus oryzae*, reveals a whole genome duplication and large gene family expansion. Submitted

Pariseau, B., Nehls, S., Ogawa, G.S.H., Sutton, D.A., Romanelli, A.M., and **B.L. Wickes**. 2009. *Beauveria* keratitis and biopesticides: A morphological and molecular comparison. Submitted.

Hickey, P.W., Sutton, D.A., Fothergill, A.W., Rinaldi, M.G., **Wickes, B.L**., Schmidt, H.J., and T.J. Walsh. 2009. *Trichosporon mycotoxinivorans*: A Novel Respiratory Pathogen in Patients With Cystic Fibrosis. Submitted

Suedmeyer, W.K., G. L. Takle, E. Stapp, A. M. Romanelli, and **B. L. Wickes**. 2009. *Microsporum gypseum* infection in two bat eared foxes (*Otocyon megalotis*). (Submitted)

Herrera, M.L., Vallor. A.C., Patterson, T.F., and **B.L. Wickes**. 2009. Strain-dependent variation of 18s rDNA copy number in *Aspergillus fumigatus*. In Press. J. Clin. Mic.

Hurst, S.F., Romanelli, A.M., **Wickes , B.L.**, Seah, C., Zhang, S., Cuenca-Estrella, M., Tudela, J. L. R., Castelli, J. L. R., Mellado, E., Kidd, S., Morrissey, O., Simmon, K., Petti, C., Snelders, E., Verweij, P., de Valk, H., Klassen, C., and S.A. Balajee. 2009. Interlaboratory reproducibility of a single locus sequence-based method for strain typing of *Aspergillus fumigatus*. In Press J. Clin. Mic.

Rajeev, S., Sutton, D.A., **Wickes, B.L**., Miller, D.L., Giri, D., Van Meter, M., Thompson, E.H., Rinaldi, M.G., Romanelli, A.M., Cano, J.F., and J. Guarro. 2009. Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). In Press. J Clin Microbiol.

Balajee, A., A. Borman, M. Brandt, J. Cano, M. Cuenca-Estrella, E. Dannaoui, J. Guarro, G. Haase, C. Kibbler, W. Meyer, K. O'Donnell, C. Petti, J. Rodriguez-Tudela, D. Sutton, A. Velagraki, and **B.L. Wickes**. 2009. Sequence-based identification of *Aspergillus, Fusarium* and *Mucrorales* in the routine mycology laboratory: Where are we and where should we go from here? J. Clin. Mic. (In Press)

Nyaoke, A. E., Weber, S., Innis, C., Stremme, D., Dowd, C., Hinckley, L., Gorton, T., **Wickes, B.L**., Sutton, D., de Hoog, S., and S. Frasca Jr. 2009. Disseminated phaeohyphomycosis in weedy seadragons, *Phyllopteryx taeniolatus*, and leafy seadragons, *Phycodurus eques*, caused by species of *Exophiala*, including a novel species. J. Vet. Diag. Invest. 21:69-79.

Grant, D.C., Sutton, D.A., Sandberg, C.A., Tyler, R.D., Jr., Thompson, E.H., Romanelli, A.M., and **B.L. Wickes**. 2009. Disseminated *Geosmithia argillacea* infection in a German Shepherd Dog. Med. Mycol. 47:221-226.

Sutton, D.A., **Wickes, B.L**., Thompson, E.H., Rinaldi, M.G., Roland, R.M., Libal, M.C., Russell, K., and S. Gordon. 2008. Pulmonary *Phialemonium curvatum* phaeohyphomycosis in a Standard Poodle dog. Med. Mycol. 46:355-359.

From last two reporting periods

Drees, M., **Wickes, B.L**., Gupta, M., and S. Hadley. 2007. *Lecythophora mutabilis* prosthetic valve Endocarditis in a diabetic patient. Med. Mycol. 45:463-467

Bar-Meir, M., Sutton, D.A., **Wickes, B.L**., Kurtzman, C.P., Goldman, S., and X. Zheng. 2006. Catheter-Related Fungemia due to *Candida thermophila*. J Clin Microbiol. 44:3035-6.

VI. Abstracts

1. Sutton, DA**, Wickes, B.L.**, Romanelli AM, Rinaldi MG, Thompson EH, Dishop MK, Elidemir O, Mallory GB, Moonnumakal SP, Schecter MG. 2009. Fatal Central Nervous System *Aspergillus granulosus* in a Lung Transplant Recipient. ISHAM Tokyo, Japan.

2. Ames, J.C. , Vallor, A.C. , Herrera, M.L., Erlandsen, J.E., Kirkpatrick, W.R., **Wickes, B.L**., Patterson, T.F., and S.W. Redding. *Candida dubliniensis* identification; comparing traditional microbiological to molecular methods. American Association of Dental Research Meeting, Dallas TX 2008.

3. Herrera, M.L., Vallor, A.C., Kirkpatrick, W.R., Najvar, L.K., Patterson , T.F. and **B.L. Wickes.** 2008. Strain-Dependent Variation of 18s rDNA Copy Number in *Aspergillus fumigatus.* 48th ICAAC - Interscience Conference on Antimicrobial Agents. Washington, D.C.

4. Erlandsen, J.E., Vallor, A.C., Kirkpatrick, W.R., Herrera, M.L., **Wickes, B.L**., Berg, D.L., Westbrook, S.D., Redding, S.W., and TF Patterson. 2008. Current Prevalence of *Candida* spp. Colonization, Fluconazole (FLU) Susceptibility and Oropharyngeal Candidiasis (OPC) in HIV/AIDS Patients Using Combined Microbiological and Molecular Methods of Detection. 48th ICAAC - Interscience Conference on Antimicrobial Agents. Washington, D.C.

5. Romanelli, A.M., and **B.L. Wickes**. 2007. Development of a Biocurated Sequence Identification Database for Molecular Identification of Human Fungal Pathogens. 5th Annual South Central Medical Mycology Meeting. San Antonio, TX.

6. Vallor, A.C., W.R. Kirkpatrick, J.E. Erlandsen, D.K. Berg, S.D. Westbrook, W. Sanns, M. Herrera, **B.L. Wickes**, S.W. Redding, and T.F. Patterson. 2007. *In Vivo* Detection of Resistant Yeasts and Significance in Orophayngeal Candidiasis (OPC) in AIDS Patients in the Era of HAART. 47th ICAAC - Interscience Conference on Antimicrobial Agents. Chicago, IL

7. Nyaoke, A., E.S. Weber, C. Innis, D. Stremme, C. Dowd, C. Keller, L. Hinckley, T. Gorton, **B.L. Wickes**, E. Thompson, D. Sutton, S. de Hoog, and S. Frasca Jr. 2007. Species Identification of *Exophiala* from investigations of phaeohyphomycosis in aquaria. Annual Conference of the American Association of Veterinary Laboratory Diagnosticians. Reno, Nevada

8. Sutton, D., E. Thompson, M. Rinaldi, A. Romanelli and **B.L. Wickes**. 2007. Lack of Correlation between ITS and D1D2 rDNA Sequence Data for the Molecular Identification of Clinical Filamentous Basidiomycetes. 47th ICAAC - Interscience Conference on Antimicrobial Agents. Chicago, IL

9. Nyaoke, A., E.S. Weber, C. Innis, D. Stremme, C. Dowd, C. Keller, L. Hinckley, T. Gorton, **B. Wickes**, D. Sutton, S. de Hoog, and S. Frasca Jr. 2007. Advances In Our Understanding Of Phaeohyphomycosis. 32nd Eastern Fish Health Workshop. Gettysburg, Pa.

10. Herrera, M. and **B.L. Wickes**. 2006. Ribosomal RNA copy number is strain dependent in *Aspergillus fumigatus*: Implications for qRT-PCR. 4th Annual South Central Medical Mycology Meeting. Houston, TX.

CONCLUSION: The third year of this study prepared the database for use by populating it with Type culture sequences and additional clinical isolate sequences. We have been testing the database by using it to identify clinical isolates that come into the Fungus Testing Laboratory (Dept. of Pathology, UTHSCSA). Since the database is operational, we have added functionality to it through the use of new fields, and also created a second database that will allow us to rapidly identify and add isolates that we are interested in. We have also have begun to apply our database to the main purpose of this proposal, which is a military application. Toward this end we have initiated a collaboration with investigators at Brooks Army Medical Center, who are

sending us clinical isolates from soldiers who are burn patients to test our database with. We hope to expand this collaboration in the future. A second collaboration is being discussed with investigators at Walter Reed Army Medical Center, Washington, DC, and The Uniformed Services University, Bethesda. These results have now positioned our study for the last task, which is a comparative study of our approach to identification with standard clinical approaches.

REFERENCES:

1. Sutton, D.A., **Wickes, B.L.**, Romanelli, A.M., Rinaldi, M.G., Thompson, E.H., Fothergill, A.W., Dishop, M.K., Elidemir, O., Mallory, G.B., Moonnamakal, S.P., Adesina, A.M., and M.G. Schecter. 2009. Fatal Central Nervous System *Aspergillus granulosus* in a Lung Transplant Recipient. Submitted.

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4. Readinger, A., LaSala, P.R., McGinnis, M.R., **Wickes, B.L**., Sutton, D.A., and M.G. Wilkerson. 2009. Hyalohyphomycosis caused by the coelomycete *Microsphaeropsis arundinis* in a cadaveric renal transplant patient. Submitted

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8. Pariseau, B., Nehls, S., Ogawa, G.S.H., Sutton, D.A., Romanelli, A.M., and **B.L. Wickes**. 2009. *Beauveria* keratitis and biopesticides: A morphological and molecular comparison. Submitted.

9. Hickey, P.W., Sutton, D.A., Fothergill, A.W., Rinaldi, M.G., **Wickes, B.L**., Schmidt, H.J., and T.J. Walsh. 2009. *Trichosporon mycotoxinivorans*: A Novel Respiratory Pathogen in Patients

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11. Herrera, M.L., Vallor. A.C., Patterson, T.F., and **B.L. Wickes**. 2009. Strain-dependent variation of 18s rDNA copy number in *Aspergillus fumigatus*. In Press. J. Clin. Mic.

12. Hurst, S.F., Romanelli, A.M., **Wickes , B.L.**, Seah, C., Zhang, S., Cuenca-Estrella, M., Tudela, J. L. R., Castelli, J. L. R., Mellado, E., Kidd, S., Morrissey, O., Simmon, K., Petti, C., Snelders, E., Verweij, P., de Valk, H., Klassen, C., and S.A. Balajee. 2009. Interlaboratory reproducibility of a single locus sequence-based method for strain typing of *Aspergillus fumigatus*. In Press J. Clin. Mic.

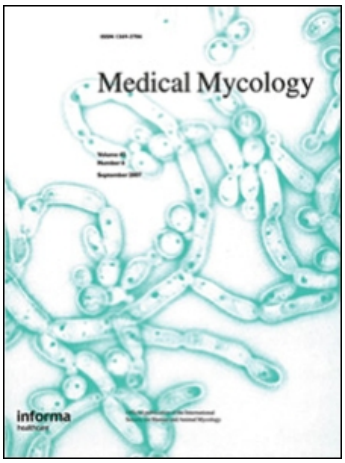
13. Rajeev, S., Sutton, D.A., **Wickes, B.L**., Miller, D.L., Giri, D., Van Meter, M., Thompson, E.H., Rinaldi, M.G., Romanelli, A.M., Cano, J.F., and J. Guarro. 2009. Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). In Press. J Clin Microbiol.

14. Balajee, A., A. Borman, M. Brandt, J. Cano, M. Cuenca-Estrella, E. Dannaoui, J. Guarro, G. Haase, C. Kibbler, W. Meyer, K. O'Donnell, C. Petti, J. Rodriguez-Tudela, D. Sutton, A. Velagraki, and **B.L. Wickes**. 2009. Sequence-based identification of *Aspergillus, Fusarium* and *Mucrorales* in the routine mycology laboratory: Where are we and where should we go from here? J. Clin. Mic. (In Press)

15. Nyaoke, A. E., Weber, S., Innis, C., Stremme, D., Dowd, C., Hinckley, L., Gorton, T., **Wickes, B**.**L**., Sutton, D., de Hoog, S., and S. Frasca Jr. 2009. Disseminated phaeohyphomycosis in weedy seadragons, *Phyllopteryx taeniolatus*, and leafy seadragons, *Phycodurus eques*, caused by species of *Exophiala*, including a novel species. J. Vet. Diag. Invest. 21:69-79.

16. Grant, D.C., Sutton, D.A., Sandberg, C.A., Tyler, R.D., Jr., Thompson, E.H., Romanelli, A.M., and **B.L. Wickes**. 2009. Disseminated *Geosmithia argillacea* infection in a German Shepherd Dog. Med. Mycol. 47:221-226.

17. Sutton, D.A., **Wickes, B.L**., Thompson, E.H., Rinaldi, M.G., Roland, R.M., Libal, M.C., Russell, K., and S. Gordon. 2008. Pulmonary *Phialemonium curvatum* phaeohyphomycosis in a Standard Poodle dog. Med. Mycol. 46:355-359.



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Pulmonary Phialemonium curvatum phaeohyphomycosis in a Standard Poodle dog

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Pulmonary *Phialemonium curvatum* phaeohyphomycosis in a Standard Poodle dog

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Phialemonium curvatum, frequently misidentified as an *Acremonium* species, is reported here as a new agent of pulmonary phaeohyphomycosis in a Standard Poodle dog, and added as a new species in the genus to cause mycoses in canines. *In vitro* susceptibility data, for both human and animal isolates, suggests resistance to amphotericin B and susceptibility to the triazole agents itraconazole, voriconazole, and posaconazole.

Keywords *Phialemonium curvatum*, canine, systemic phaeohyphomycosis

Introduction

Systemic phaeohyphomycosis, a disease associated with saprobic dematiaceous fungi, has been reported infrequently in the dog. In humans *Cladophialophora bantiana* (synonyms, *Cladosporium trichoides, Cladosporium bantianum, Torula bantiana, Xylohypha bantiana, Xylohypha emmonsii*) is known to be neurotropic, and animals with systemic phaeohyphomycosis also commonly present with neurologic disease. In four reports of systemic *C. bantiana* infection in dogs the animals presented with a clinical history and/or signs of central nervous disease including tetraparesis, neck stiffness, back pain, circling, opisthotonus, nystagmus, protrusion of the nictitating membrane and/or seizures [1–4]. *Ochroconis gallopavum* [5] and *Aureobasidium pullulans* [6] have been isolated from lesions in dogs presenting with ataxia, seizures or 'neurologic dysfunction' and in another case dematiaceous fungi were demonstrated in the brain of an animal presenting with convulsions [7]. Rarely, animals with phaeohyphomycosis present with other primary clinical disease, as in the pug dog with a chronic skin infection and who had a dual systemic infection caused by *Bipolaris spicifera* and *Candida* (*Torulopsis*) *glabrata* [8]. Most cases of systemic phaeohyphomycosis in the dog have been diagnosed at necropsy. In the case reported here, the etiologic agent of pulmonary disease was detected antemortem.

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Case report

A two-year-old male, castrated Standard Poodle was presented to the Texas A&M University Veterinary Medical Teaching Hospital in August, 2005 for definitive surgical repair of an atrial septal defect. The surgery was successful. Several days post-operatively the dog developed vasculitis, pancreatitis, as well as pneumonia, which were treated with palliative therapy in combination with enrofloxacin and ticarcillin/clavulanic acid. In October, 2005 the dog developed a chylous pleural effusion, which was corrected by surgical ligation of the thoracic duct in November 2005. The chylous effusion was thought to represent a complication of his previous open heart surgery. Following this surgery, the dog developed pneumonia again as well as a serosanguinous pleural effusion. The pneumonia was treated with enrofloxacin as well as amoxicillin/clavulanic acid. The pleural effusion became chronic and frequent thoracocenteses were performed to help control the clinical signs associated with the effusion. In December, 2005 a course of somatostatin was given but failed to resolve the effusion.

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Subsequently immunosuppressive prednisone therapy was initiated. After the addition of prednisone, the dog remained asymptomatic for his pleural effusion for eight months, during which the dose was gradually reduced to an anti-inflammatory dose range and the severity of the effusion was monitored with thoracic radiographs and echocardiography. Pleurocentensis was not performed at anytime during this 8 month period, after which the dog developed dyspnea and a severe suppurative pleural effusion. In addition, right rear lameness developed and a right carpal joint tap revealed septic, suppurative effusion. A urinary tract infection was also documented at this time. The dog was treated with thoracocentesis as needed to control clinical signs of dyspnea and a combination of ticarcillin/clavulanic acid, amoxicillin/clavulanic acid, and enrofloxacin. The prednisone was continued although doses were tapered. Over the next few months the dog presented multiple times with dyspnea due to pleural effusion and thoracentesis was performed. The cytologic examinations of pleural fluid samples varied slightly during this time period with the fluid being classified as either a transudate (low cell counts and low total protein concentration) or a modified transudate (mildly increased cell count or total protein concentration), depending on total nucleated cell counts and total protein concentrations. Several bacterial cultures of the pleural fluid yielded no growth. Given the severity of the recurrent pleural effusion in the face of reducing prednisone doses, and the historical response of the pleural effusion to immunosuppressive doses of prednisone, azothioprine was added to the drug regimen in an attempt to further immunosuppress the patient, and potentially allow the dose of prednisone to be reduced. Azothioprine was not well tolerated and was discontinued. Prednisone was continued at immunosuppressive doses and antibiotic coverage with a combination of clavamox and enrofloxacin were continued. Intermittent pleurocentesis for symptomatic pleural effusion continued although the frequency was somewhat reduced. In October, 2006 the animal was presented again with dyspnea and severe pleural effusion. New skin lesions had also developed near the right carpal footpad and over both tarsi. Cytologic examination of pleural fluid revealed a modified transudate/hemorrhagic effusion and cytologic examination of the footpad lesion revealed mild inflammation with intracellular fungal elements present. In addition, multiple lesions suggestive of dermatophytosis developed on the skin of the inguinal area. Three bacterial cultures and one fungal culture were inoculated with pleural fluid samples (collection dates 27 October 2006, 14 November 2006, 22 November 2006 and 3 November 2006 respectively). In addition, fungal cultures were started with samples from the footpad and tarsal lesions (collection date 14 November 2006). Pleural fluid samples for bacteria were inoculated onto trypticase soy agar with 5% sheep blood, MacConkey's agar, and into tryptose broth (Becton Dickinson, Sparks, MD) and incubated at 37°C in 5% CO_2 for up to 5 days. The first two pleural fluid samples yielded no bacterial growth however a fungus was isolated on the blood agar plates inoculated with each of these samples after 3 and 4 days of incubation, respectively. The third pleural fluid sample yielded *Staphylococcus aureus* after one day of incubation and a fungus on day 5 of incubation. The fungal isolates cultured from pleural fluid on three separate occasions appeared identical. One pleural fluid sample inoculated onto only Sabouraud dextrose agar ([SDA], (BD, BBL, Sparks, MD) was negative after 18 days incubation at 25°C. *Microsporum gypseum* was recovered in cultures started with the tarsal skin samples. The footpad culture grew a sterile dematiaceous mould that was subsequently identified as a coelomycete morphologically resembling a *Microsphaeropsis arundinis* (distinct from the fungi isolated from the pleural fluid samples). At this time azathioprine was discontinued and the dog was started on amphotericin B. The dog then developed a methicillin-resistant *Staphylococcus aureus* infection. Due to the poor prognosis and deteriorating condition, the dog was euthanized in December 2006. Necropsy was not performed.

Identification of the etiologic agent

Three fungal isolates from pleural fluid samples and the isolate from the footpad were forwarded to the Fungal Testing Laboratory at University of Texas, San Antonio, TX for identification. The pleural fluid isolates were accessioned into their stock collection as UTHSC 06-4324, 06-4325, and 06-4326. The morphologic features of the isolates were examined on in house prepared potato flakes agar (PFA) incubated at 25°C. Growth rate was moderate and after two weeks incubation colonies were white to cream, floccose, effuse, with centrally raised areas. Discrete, moist, salmon to brownish-yellow sporodochial areas (macroscopically visible cushion-like masses of short conidiophores bearing conidia) formed throughout the cultures after 4 weeks incubation (Fig. 1 – taken at 8 weeks). Microscopically, hyaline hyphae produced numerous coils and complex fasicles (bundles of hyphae). Conidiogenous cells consisted primarily of adelophialides (short phialides lacking a basal septum) produced directly on the hyphae (Fig. 2) and from coils.

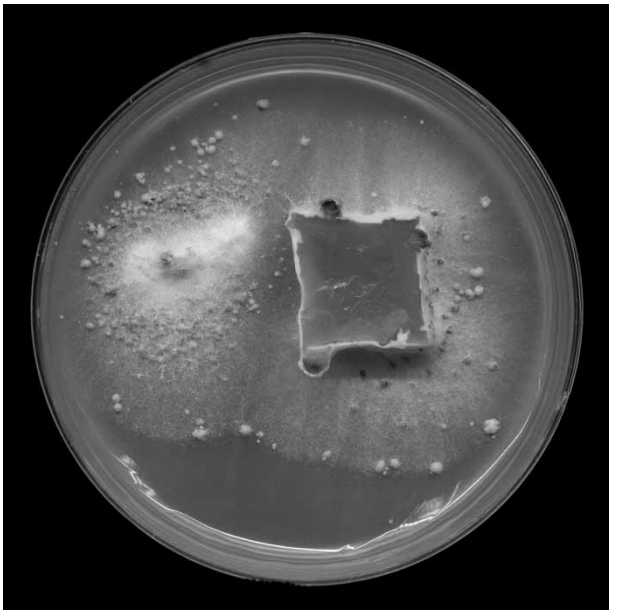

Fig. 1 Potato flakes agar plate, 8 weeks at 25°C, showing area of slide culture preparation on the right, and an undisturbed colony on the left. Salmon to brownish-yellow, moist, raised sporodochial areas are seen throughout the culture.

However longer phialides delimited by a basal septum as seen in *Acremonium* species were also occasionally present. Long, setae-like phialides were also produced from the sporodochia. Slightly allantoid (curved) conidia (1–1.5 × 4.4.5 μm) were borne in mucoid clusters at the apices of these conidiogenous cells. Chlamydoconidia were also present. Based on the features noted above, the isolate was morphologically identified as *Phialemonium curvatum* [9–11].

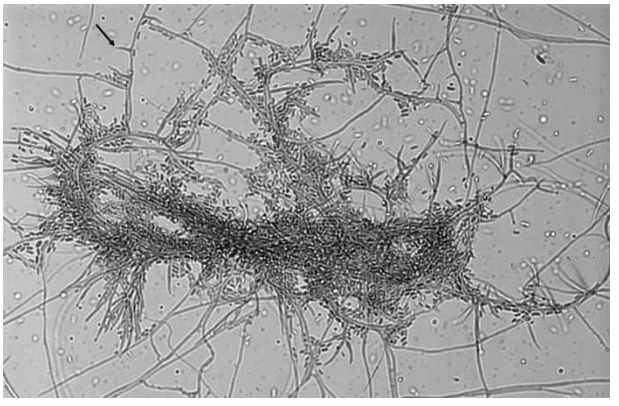

Fig. 2 Microscopic morphology of a young, immature sporodochium after 7 days growth at 25°C on potato flakes agar. Figure depicts short adelophialides (reduced phialides lacking a basal septum), black arrow, as well as longer phialides delimited by basal septa as seen in *Acremonium* species.

One of the isolates, UTHSC 06-4324 (= R-3884) was submitted for molecular characterization to confirm the morphologic identification. DNA was isolated from conidia recovered from a 72 h PDA plate using the Prepman Ultra reagent (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Five microliters each of the supernatant were used in two PCR reactions to amplify the ITS and D1/D2 regions from the rDNA locus. The ITS region was amplified as described using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [12]. The D1/D2 region was amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3) as described [13,14]. Both PCR reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) using Triple Master Taq polymerase (Fisher Scientific, Pittsburgh, PA). Amplicons were purified using a Qiaquick PCR purification kit (Qiagen, Inc., Valencia, CA) and then sequenced on both strands at the UTHSCSA Advanced Nucleic Acids Core facility. The data obtained from each sequence were then used to perform BLASTn searches at the NCBI website <http://ncbi.nlm.nih.gov/BLAST/> [15]. Identifications were made at a cutoff of ≥ 98% sequence identity.

The results of the two BLAST searches showed the greatest identity with other sequences deposited from *P. curvatum*. The top three hits for the D1/D2 sequence were *P. curvatum* sequences, each at 99% identity. The top three hits for the ITS search were *P. dimorphosporum*, *P. curvatum*, and *P. curvatum*, each at 99% identity. Since *P. dimorphosporum* is a synonym of *P. curvatum* [16], the sequence identity of the isolate was assigned as *P. curvatum*. The case isolate UTHSC 06-4323 (= R-3884) has been deposited in the University of Alberta Microfungus Collection under the accession number UAMH 10825. The nucleotide sequence data has been deposited into GenBank under the accession numbers EU035984 (ITS) and EU035985 (D1/D2).

The footpad isolate was accessioned as UTHSC 06-4327. After one month incubation at 25°C on a variety of media prepared in-house including PDA, V-8 agar, and carnation leaf agar [17], rare pycnidial structures developed. Conidia were narrow-cylindrical, 1–1.5 × 4 μm, individually subhyaline, but dark in mass. Based on these features the isolate morphologically resembled the coelomycete *Microsphaeropsis arundinis*. The recovery of this organism from the footpad, while potentially significant for localized infection at this site [18,19], was not contributory to systemic fungal

disease. No additional testing was performed on this isolate.

In vitro antifungal susceptibility testing

Retrospective antifungal susceptibility testing of *P. curvatum* was accomplished in a macrobroth dilution format in essential agreement with the previously published Clinical and Laboratory Standards Institute document M38-A [20]. Amphotericin B (AMB, Bristol-Meyers, Squibb, New York, NY) and caspofungin (CAS, Merck, Rahway, NJ) were tested in Antibiotic Medium 3 (Difco, Sparks, MD) while 5-fluorocytosine (5FC, Valient, Irvine, CA), fluconazole, voriconazole (FLC, VRC, Pfizer, Inc., New York, NY), itraconazole (ITC, Janssen Pharmaceutica, Piscataway, NJ) and posaconazole (PSC, Schering Plough, Galloping Hill, NJ) were tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Tubes were incubated at 35°C with endpoints read at 24 and 48 h. The endpoints for AMB were the lowest concentration that inhibited visual growth, while those for 5FC and the triazoles were 80% inhibition compared to the growth control. Caspofungin endpoints were read as minimum effective concentrations (MECs) [21,22]. Results at 24/48 h were as follows in μg/ml: AMB 2/4; CAS 0.25/0.5; 5-FC >64; FLC 8/16; ITC 0.06/0.25; VRC 0.125/0.25; PSC 0.03/0.125.

Discussion

The genus *Phialemonium,* having morphologic features between the genera *Acremonium* and *Phialophora,* currently contains two species, *P. obovatum* and *P. curvatum* [9,16]. *Phialemonium obovatum* produces a distinct, pale green diffusing pigment, has obovate conidia (like an upside-down egg), and has been previously reported in German shepherd dogs causing osteolytic [23] and disseminated disease [24]. To our knowledge, this is the first report of *P. curvatum* in the veterinary literature. As the use of SDA as a sole primary isolation medium is less than optimal for the recovery of filamentous fungi, this and other etiologic agents may be under-diagnosed. Sporodochial-forming *Phialemonium curvatum* isolates were initially recognized in 2004 in human cases of hemodialysis-associated endovascular infection [10]. They have subsequently been seen in cases of endocarditis and endophthalmitis stemming from intracavernous penile autoinjections of contaminated fluids [11,25], and from intra-articular injection of corticosteroids [26]. Isolates are often misidentified as *Acremonium* species based on the overall macroscopic and microscopic similarities of the two genera.

In humans, the formation of phialides and phialoconidia within tissues in the host, termed 'adventitious' conidia by Liu *et al*. [27], appear to facilitate hematogenous dissemination inciting fungemia [16], endocarditis [28,29], and peritonitis [30]. Disseminated disease usually occurs in the setting of immune compromise. The same scenario presumably occurs in dogs. On occasion dogs operated for chylous effusion can develop non-chylous effusion post operatively that responds to immunosuppression suggesting an underlying inflammatory etiology [31]. However, dogs receiving chronic immunosuppressive agents are, like humans, at risk for infections, particularly from a variety of potential fungal pathogens [32,33]. Secondary infections may also be present and, in this case, likely contributed to the severity of the pleural effusion in the later stages of the disease.

Retrospective antifungal susceptibility results for the case isolate were similar to those seen for human isolates. Although there are no defined breakpoints for this organism, elevated MICs for AMB and 5FC suggested resistance. Clinical deterioration while on AMB therapy may support lack of efficacy for this agent. Itraconazole, as well as the newer triazoles PCZ and VCZ, demonstrated low MICs, while the FLZ MIC was somewhat elevated at 16μg/ml. Minimum effective concentrations for CAS were also low at 0.5 μg/ml; a departure from MEC values seen in human isolates.

In conclusion, *Phialemonium curvatum* is reported as a new agent of pulmonary phaeohyphomycosis in a Standard Poodle dog, and is added as a new species in the genus to cause disease in canines. Based on limited data, the triazole drugs ITZ, VRZ, and PCZ would appear appropriate for empiric therapy pending susceptibility test results.

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References

1 Anor S, Sturges BK, Lafranco K, *et al*. Systemic phaeohyphomycosis (*Cladophialophora bantiana*) in a dog – clinical diagnosis with sterotactic computed tomographic-guided brain biopsy. *J Vet Intern Med* 2001; **15**: 257–261.

2 Dillehay DL, Ribas JL, Newton JC, Kwapien RP. Cerebral phaeohyphomycosis in two dogs and a cat. *Vet Path* 1987; **24**: 192–194.

3 Schroeder H, Jardine JE, Davis V. Systemic phaeohyphomycosis caused by *Xylohypha bantiana* in a dog. *J S Afr Vet Assoc* 1994; **65**: 175–178.

4 Fiske RA, Choyce PD, Whitford HW, Martin EC. Phaeohyphomycotic encephalitis in two dogs. *J Am Anim Hosp Assoc* 1986; **22**: 327–330.

5 Singh K, Flood J, Welsh RD, *et al*. Fatal systemic phaeohyphomycosis caused by *Ochroconis gallopavum* in a dog (*Canis familaris*). *Vet Path* 2006; **43**: 988–992.

6 Perkins MC, Martin P, Krockenberger MB, Weeks K, Malik R. Cerebral phaeohyphomycosis in a young female German shepherd. *Australian Vet Prac* 2004; **34**: 110.

7 Migaki G, Casey HW, Bayles WB. Cerebral phaeohyphomycosis in a dog. *J Am Vet Med Assoc* 1987; **191**: 997–998.

8 Waurzyniak BJ, Hover JP, Clinkenbeard KD, Welsh RD. Dual systemic mycosis caused by *Bipolaris spicifera* and *Torulopsis glabrata* in a dog. *Vet Path* 1992; **6**: 566–569.

9 Gams W, McGinnis MR. *Phialemonium*, a new anamorph genus intermediate between *Phialophora* and *Acremonium*. *Mycologia* 1983; **75**: 977–987.

10 Proia LA, Hayden MK, Kammeyer PL, *et al*. *Phialemonium* species: Emerging mold pathogens that caused 4 cases of hemodialysis-associated endovascular infection. *Clin Infect Dis* 2004; **39**: 373–379.

11 Weinberger M, Mahrshak I, Keller N, *et al*. Isolated endogenous endophthalmitis due to a sporodochial-forming *Phialemonium curvatum* acquired through intracavernous autoinjections. *Med Mycol* 2006; **44**: 253–259.

12 White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand D, Sninsky J, White TC (eds). *PCR-Protocols and Applications – A Laboratory Manual*. New York, NY: Academic Press, 1990: 315–322.

13 Kurtzman CP, Robnett CJ. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* 1997; **35**: 1216–1223.

14 Peterson SW, Kurtzman CP. 1990. Phylogenetic relationships among species of the genus *Issatchenkia* Kudriavzev. *Antonie Van Leeuwenhoek* 1990; **58**: 235–240.

15 Altschul SF, Madden TL, Schäffer AA, *et al*. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389–3402.

16 Guarro J, Nucci M, Akiti T, *et al*. *Phialemonium* fungemia: two documented nosocomial cases. *J Clin Microbiol* 1999; **37**: 2493–2497.

17 Nelson PE, Toussoun TA, Marasas WFO. 1983. *Fusarium species. An Illustrated Manual for Identification*. Pennsylvania State University Press: University Park, PA.

18 Pendle S, Weeks K, Priest M, *et al*. Phaeohyphomycotic soft tissue infections caused by the coelomycetous fungus *Microsphaeropsis arundinis*. *J Clin Microbiol* 2004; **42**: 5315–5319.

19 Kluger EK, Della Torre PK, Martin P, Krockenberger MB, Malik R. Concurrent *Fusarium chlamydosporum* and *Microsphaeropsis* arundinis infections in a cat. *J Feline Med Surg* 2004; **6**: 271–277.

20 Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. CLSI document M38-A. 2002; Clinical and Laboratory Standards Institute, Wayne, PA.

21 Arikan S, Lozano-Chiu M, Paetznick V, Rex JH. *In vitro* susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob Agents Chemother* 2001; **45**: 327–330.

22 Kurtz MB, Heath IB, Marrinan J, *et al*. Morphological effect of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-beta-D-glucan synthase. *Antimicrob Agents Chemother* 1994; **38**: 1480–1489.

23 Lomax LG, Cole JR, Padhye AA, *et al*. Osteolytic phaeohyphomycosis in a German shepherd dog caused by *Phialemonium obovatum*. *J Clin Microbiol* 1996; **23**: 987–991.

24 Smith AN, Spencer JA, Stringfellow JS, Vygantas KR, Welch JA. Disseminated infection with *Phialemonium obovatum* in a German shepherd dog. *J Am Vet Med Assoc* 2000; **216**: 708–712.

25 Strahilevitz J, Rahav G, Schroers H-J, *et al*. An outbreak of *Phialemonium* infective endocarditis linked to intracavernous penile injection for the treatment of impotence. *Clin Infect Dis* 2005; **40**: 781–786.

26 Dan M, Yossepowitch O, Hendel D, Shwartz O, Sutton DA. *Phialemonium curvatum* arthritis of the knee following intra-articular injection of a corticosteroid. *Med Mycol* 2006; **44**: 571–574.

27 Liu K, Howell DN, Perfect JR, Schell WA. Morphologic criteria for the preliminary identification of *Fusarium*, *Paecilomyces*, and *Acremonium* species by histopathology. *Am J Clin Pathol* 1997; **109**: 45–54.

28 Schonheyder HC, Jensen HE, Gams W, *et al*. Late bioprosthetic valve endocarditis caused by *Phialemonium curvatum* and *Streptococcus sanguis*: a case report. *J Med Vet Mycol* 1996; **34**: 206–214.

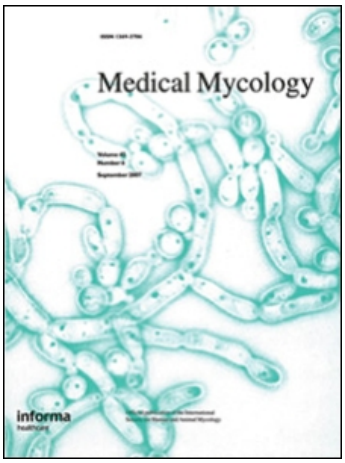
29 Gavin PJ, Sutton DA, Katz BZ. Fatal endocarditis caused by the dematiaceous fungus *Phialemonium obovatum*: case report and review of the literature. *J Clin Microbiol* 2002; **40**: 2207–2212.

30 King D, Pasarell L, Dixon DM, McGinnis MR, Merz WG. A phaeohyphomycotic cyst and peritonitis caused by *Phialemonium* species and a re-evaluation of its taxonomy. *J Clin Microbiol* 1993; **31**: 1804–1810.

31 Smeak DD, Gallagher L, Birchard SJ, Fossum TW. Management of intractable pleural effusion in a dog with pleuroperitoneal shunt. *Vet Surg* 1987; **16**: 212–216.

32 Lionakis MS, Kontoyiannis DP. Glucocorticoids and invasive fungal infections. *The Lancet* 2003; **362**: 1828–1838.

33 Grooters AM. Weird fungi: Making sense of emerging and unusual mycoses. In: Proceedings of the 21st Annual American College of Veterinary Internal Medicine Forum; Charlotte, NC, 2003.



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Disseminated Geosmithia argillacea infection in a German Shepherd dog

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Disseminated *Geosmithia argillacea* infection in a German Shepherd dog

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We report a systemic mycosis in a German Shepherd dog caused by *Geosmithia argillacea*. Although this etiologic agent microscopically resembles a *Penicillium* species, and is histopathologically compatible with members of the genus *Aspergillus*, morphologic features and molecular characterization clearly separate it from these genera. This appears to be the first report of disseminated disease by this species in humans or animals. *In vitro* antifungal susceptibility testing suggests resistance to amphotericin B and voriconazole and susceptibility to caspofungin, itraconazole, and posaconazole.

Keywords *Geosmithia argillacea*, German Shepherd dog

Introduction

Disseminated opportunistic mycoses are infrequently reported in dogs. The most common etiologic agents are species of *Aspergillus,* particularly *A. terreus* and *A. deflectus* [1–12]. There are rare reports of disseminated disease caused by other hyaline genera such as *Penicillium* [13]*, Paecilomyces* [14], *Sagenomella* [15], and agents of adiaspiromycosis [16], as well as isolated reports of systemic phaeohyphomycosis [17]. The majority of these opportunistic infections have occurred in German Shepherd dogs leading to suspicion of a breed-related immunodeficiency, although studies by Day *et al*. failed to identify the specific defect [3]. In fact, German Shepherd male dogs have an odds ratio of 49 for disseminated aspergillosis relative to a background hospital population, and female dogs have an odds ratio of 2.9 [12]. Some of the manifestations of these disseminated mycoses in dogs have included discospondylitis, osteomyelitis, spinal hyperpathia, paralysis, pyrexia, weight loss, anorexia, uveitis, endophthalmitis, lameness, head tilt, nystagmus, renal failure, and urinary incontinence. Response to therapy with amphotericin B or triazole antifungals has been marginal. We report here the first case of disseminated infection with *Geosmithia argillacea*.

Case report

A 4-year-old female, spayed German Shepherd dog presented to the Virginia-Maryland Regional College of Veterinary Medicine in February 2008, for evaluation of acute onset glaucoma of the right eye. Moderate aqueous flare and cells, iris bombe, and preiridal membrane were noted on slit lamp biomicroscopy. Vitreal debris and exudative retinal detachment were noted on ocular ultrasonography. The intraocular pressure was 27 mmHg by rebound tonometry. Panuveitis and secondary glaucoma of the right eye were diagnosed. There were no abnormalities detected in the left eye. Topical prednisolone acetate, timolol maleate, and dorzolamide and oral carprofen were prescribed. Due to the combined presence of lethargy, spinal hyperpathia, and panuveitis, an underlying systemic disease was suspected as the cause of the ocular abnormalities and thus the dog was further evaluated. Negative antibody titer results were obtained for *Leptospira* species and *Brucella canis* (Virginia Department of Agriculture and Consumer Services, Wythe-

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ville, VA) and *Aspergillus fumigatus* (University of Tennessee Veterinary Medical Laboratory, Knoxville, TN). Urine was negative for *Blastomyces dermatitidis* antigen (MiraVista Diagnostics, Indianapolis, IN). Results of a complete blood cell count and biochemical profile were unremarkable. Hematuria and pyuria were noted, but a urine aerobic bacterial culture was negative. Thoracic radiography revealed normal cardiac and pulmonary structures. Radiography of the spine revealed osseous proliferation with concurrent lysis of the vertebral endplates of thoracic vertebrae four, five and six consistent with discospondylitis. Similar changes were noted in multiple sternebrae. Fine-needle aspirates of these sternebrae were evaluated cytologically and yielded peripheral blood only. Ultrasonography of the abdomen revealed bilateral renal pelvic dilation with all other organs appearing normal. In March 2008 the dog was evaluated for response to ocular medications and to further pursue the cause of discospondylitis. A previously undetected systolic ejection murmur was ausculted over the left heart base. Echocardiography identified a small patent ductus arteriosus, but no valvular lesions suggestive of endocarditis or cause for the ejection murmur were found. The dog was blind in the right eye with end-stage glaucoma with buphthalmos. The intraocular pressure was 50 mmHg by rebound tonometry. Rubeosis iridis and posterior and peripheral anterior synechiae of the iris were noted in the right eye. The left eye had fibrin strands in the anterior chamber and multifocal chorioretinitis in the tapetal fundus. Enucleation of the right globe for histopathologic diagnosis was performed. Fluoroscopic-guided core biopsies of multiple sternebrae were obtained. Aerobic bacterial cultures of a vitreal aspirate, sternebral biopsy, and urine were negative. Carprofen and tramadol were given post-operatively for pain control. Histopathologic evaluation of the eye identified lymphoplasmacytic panuveitis, intra-retinal hemorrhage, lens capsule rupture with pyogranulomatous inflammation, and retinal detachment with exudative vitreitis. Histopathologic evaluation of the sternebrae by hematoxylin and eosin staining revealed mild lamellar bone resorption with fibrous replacement. Fungal Gomori methenamine silver (GMS) stains revealed septate, dichotomously branching hyphae measuring 3–5 μm in diameter within the lens, retina, and sternebrae (Fig. 1 & 2). *Aspergillus terreus* was suspected based on histopathology compatible with aspergillosis and the reported prevalence of this organism in German Shepard dogs. A urine sample was obtained by cystocentesis and inoculated onto Sabouraud dextrose agar (SDA) (Remel, Lenexa, KS). The

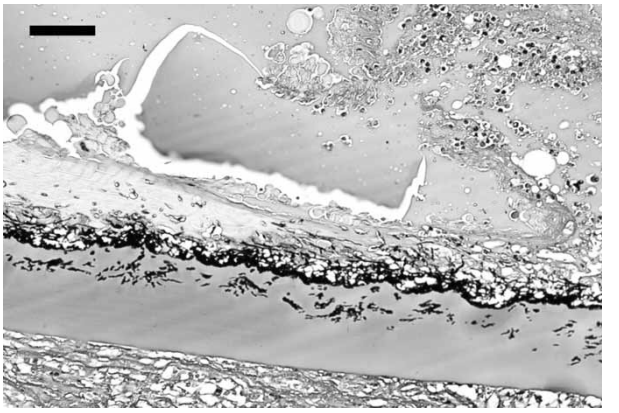

Fig. 1 GMS stain, eye, (bar equals 50 microns). Multiple septate hyphae invading the anterior lens capsule and lens cortical material.

microscopic morphology of the isolate grown on this medium after 14 days incubation at 30° C resembled a *Penicillium* species, although the isolate was subsequently identified as *Geosmithia argillacea* at the University of Texas Health Science Center at San Antonio (UTHSC). No antifungal treatment was administered. Over the next month the dog became increasingly agitated and developed a head tilt and nystagmus. Examination of the left eye revealed more severe posterior segment disease, with vitreal debris, chorioretinal scarring and focal retinal detachment. Humane euthanasia was elected and a necropsy was performed.

At necropsy the pleural surfaces were red and granular, and multiple 0.5–1mm nodules were dispersed throughout the lungs. The liver was diffusely congested and slightly enlarged. The kidneys were irregular and red and contained multifocal, small,

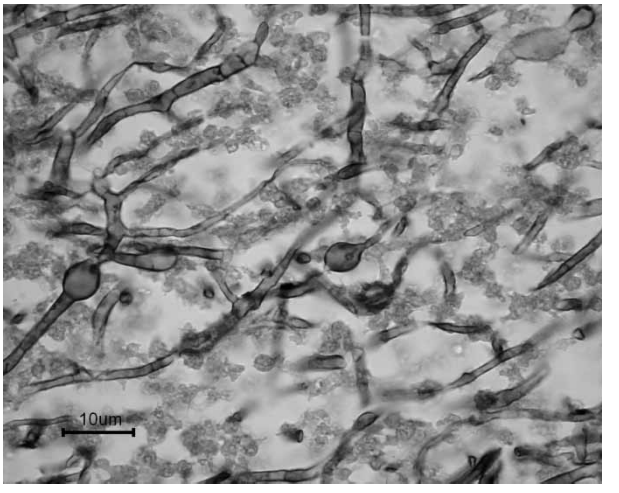

Fig. 2 GMS stain, sternebra, (bar equals 10 microns). Multiple septate hyphae with bulbous endings are dispersed throughout.

white-tan, granular nodules most prominent along the pelvises (Fig. 3). The spleen was diffusely enlarged and mottled red-white. The third, fourth, and fifth sternebrae were enlarged with a firm proliferation between the articular surfaces. The bodies of the sternebrae were osteolytic and filled with a brown-tan granular caseous material. The ventral aspects of the fifth, sixth, and seventh thoracic vertebrae were thickened with firm nodules along the articular surfaces. There was marked osteolysis of the central vertebral bodies and they were filled with a white caseous material. The right cerebrum of the brain was moderately firm but the remainder of the central nervous system was unremarkable. Microscopically the lungs, pancreas, liver, kidney, and cerebrum had multifocal regions of granulomatous inflammation often associated with blood vessels. Some granulomas from each of these organs were centrally necrotic and contained septate, dichotomously-branching fungal hyphae with bulbous ends (Fig. 4). There was extensive fibrosis around regions of inflammation within the pancreas and kidneys. The affected sternebrae and thoracic vertebral bodies also had extensive osteolysis, fibrosis, necrosis and multifocal regions of granulomatous inflammation that crossed articular surfaces. Similar hyphae were seen within necrotic regions of bone. Gomori methenamine silver stains documented hyphae in all affected tissues. Tissue samples from the left cerebrum and cerebellum, affected sternebrae and vertebrae, kidney, and bladder were inoculated onto SDA. With the exception of brain tissue, all other samples grew a fungus morphologically identical, both macroscopically and microscopically, to the urine isolate previously identified as *Geosmithia argillacea*. Molecular confirmation of the same organism from both the urine and the vertebra confirmed *Geosmithia argillacea* as the etiologic agent of disseminated disease.

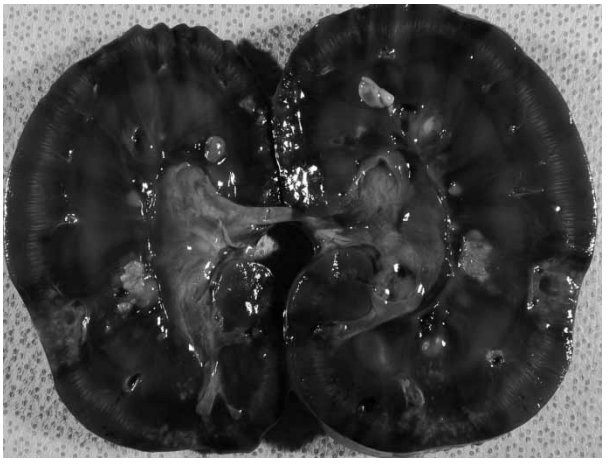

Fig. 3 The kidney is irregular and red with multifocal, large, white-tan, granular nodules most prominent along the renal pelvis. There is a wedge shaped pale area extending from the cortex to the medulla consistent with an infarct.

Identification of the etiologic agent

Both the urine isolate and the necropsy thoracic vertebra isolate were forwarded to the Fungus Testing Laboratory for molecular and morphologic characterization and were accessioned into their stock collection as UTHSC R-4148 and R-4234, respectively. Isolates were grown for 20 h at 30°C on potato dextrose agar (Difco, Detroit, MI). A small amount of hyphae was removed and suspended in 50 µl of Prepman Ultra reagent (Applied Biosystems, Foster City, CA) in a 0.5 ml microfuge tube. The suspension was heated for 15 min at 100°C and then pelleted for 5 min at 14, 000 *g* in a microfuge according to the manufacturer's instructions. PCR reactions were performed directly on 5 µl of the Prepman supernatant in a 50 µl reaction using TripleMaster *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's instructions. ITS amplicons were obtained using primers (ITS1 and ITS4) and PCR conditions as previously described [18]. D1/D2 PCR amplicons were obtained using primers (NL-1 and NL-4) and PCR conditions as described [19,20]. Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) and amplicons of the expected size were visualized by running a 15 µl aliquot of each PCR reaction on a 0.7% agarose gel followed by staining with ethidium bromide and viewed by ultraviolet transillumination. The remaining PCR template was prepared for sequencing by cleaning with a QIAquick PCR purification column (Qiagen, Valencia, CA). Purified templates were sequenced at the UTHSCSA Advanced Nucleic Acids Core facility using the same primers for ITS and D1/D2 amplification. Sequences were then used to perform individual BLASTn (Basic Local Alignment Search Tool) searches using the NCBI (National Center for Biotechnology Information) BLAST database. Genbank accession numbers were assigned as follows: R-4148 ITS, D1/D2 (ACCESSION# EU862335, ACCESSION#EU862336), R-4234 ITS, D1/D2 (ACCESSION# EU862337, ACCESSION# EU862338). A BLASTn search of the R-4148 and R-4234 ITS and D1/D2 sequences returned identical results. The three highest% identities for the ITS region were: (1) *Geosmithia argillacea* 525/541 (97%) accession #AF033389, (2) *Talaromyces eburneus* (the teleomorph of *Geosmithia argillacea*) 461/477 (96%) accession #AB176614, and (3) *Monascus fumeus*

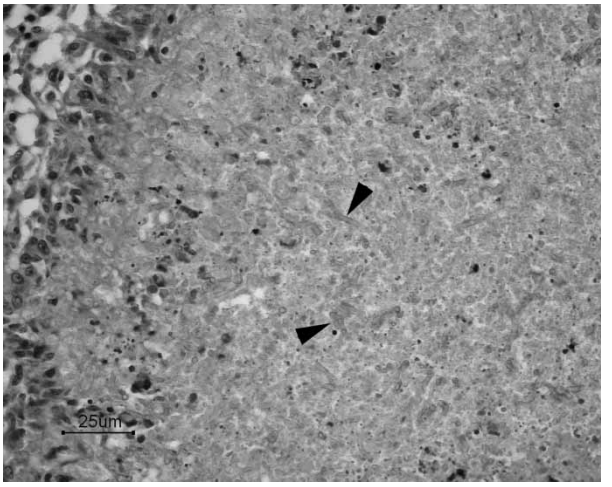

Fig. 4 H&E, kidney (bar equals 25 microns). The centers of granulomas are necrotic and contain poorly staining septate, dichotomous branching fungal hyphae (arrowheads) with bulbous endings.

508/584 (86%) accession # DQ978996. Analysis of the Genbank alignments revealed that the mismatches were in both the ITS1 and ITS2 regions. No mismatches occurred in the 5.8s rDNA region. The three highest% identities for the D1/D2 sequence were: (1) *Geosmithia argillacea* 614/614 (100%) accession # AB047236, (2) *Geosmithia argillacea* 614/614 (100%) accession # AB047235, and (3) *Geosmithia argillacea* 613/614 (99%) accession # AB047238.

The macroscopic morphology of *G. argillaceae* on malt extract agar (MEA) (Remel, Lenexa, KS, dehydrated and prepared in-house) is depicted in Fig. 5A (16 days at 23°C) and 5B (8 days at 35°C). Growth was slow and restricted at the lower temperature, attaining 21-23 mm in diameter after 16 days as compared to 34–36 mm in 8 days at the higher temperature. Colonies at 23°C were cream to buff-colored with ill-defined margins while those at 35°C were similarly colored with entire margins. Reverse and obverse colony colors were the same. Temperature studies conducted on potato flakes agar (PFA) tubed media, prepared in-house, demonstrated good growth at 37, 40, and 45°C but no growth at 50°C. Maximum growth temperatures are presumed to be near 50°C based upon our studies and those of earlier investigators [21,22]. Microscopic features observed from a PFA slide culture preparation included rough, hyaline, septate, stipes, often branched, ranging from 70–200 μm in length, penicilli that were monoverticillate to biverticillate (asymmetric) to terverticillate, cylindrical, appressed, slightly roughened phialides measuring10–12 × 2–3μm and tapering at the apex, and smooth hyaline conidia borne in long, columnar chains. Conidia, measuring 2.5–5 × 1.5–2.5 μm, were initially cylindrical to cuniform (wedge-shaped) and became ellipsoidal to ovoid at maturity (Fig. 6). Based on the sequence identities and the morphologic features, both isolates were identified as *Geosmithia argillacea* and have been deposited into the University of Alberta Mold Herbarium under the accession numbers UAMH 10932 (R-4148, urine) and UAMH 10933 (R-4234, vertebra).

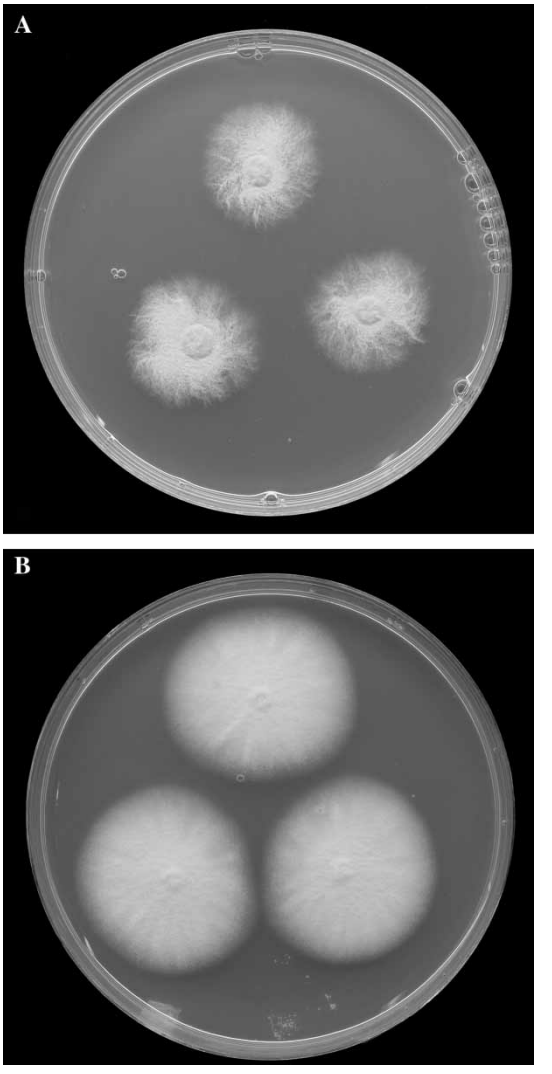

Fig. 5 Macroscopic morphology of *Geosmithia argillacea* on malt extract agar. (A) 16 days at 23°C. (B) 8 days at 35°C.

In vitro antifungal susceptibility testing

Antifungal susceptibility testing of *G. argillacea* was performed on the isolate from the vertebra. It was accomplished in a macrobroth dilution format in essential agreement with the previously published Clinical and Laboratory Standards Institute document

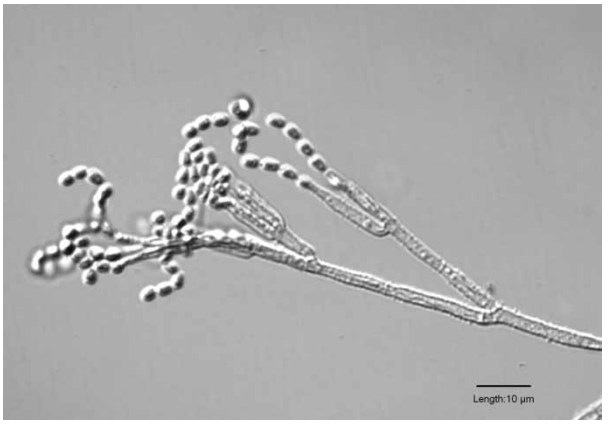

Fig. 6 Microscopic morphology of *Geosmithia argillacea* demonstrating branching stipes, monoverticillate and asymmetric biverticillate penicilli, cylindrical and appressed phialides, and smooth, hyaline, cuniform to ellipsoidal conidia borne in long, columnar chains. Roughened stipes, metulae, and phialides are a distinctive microscopic feature of this species (bar equals 10 microns).

M38-A [23]. Amphotericin B (AMB, Bristol-Meyers, Squibb, New York, NY) and caspofungin (CAS, Merck, Rahway, NJ) were tested in Antibiotic Medium 3 (Difco, Sparks, MD) while, voriconazole (VRC, Pfizer, Inc., New York, NY), itraconazole (ITC, Janssen Pharmaceutica, Piscataway, NJ) and posaconazole (PSC, Schering Plough, Galloping Hill, NJ) were tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Concentrations tested for all drugs ranged from 0.03 to 16 µg/ml. Tubes were incubated at 35°C and were read against a positive growth control tube at either 24 and 48 h (AMB and CAS) or 48 and 72 h (ITC, VRC, PSC), depending upon the growth rate of the organism in the test medium. Endpoints for AMB were the lowest concentration that inhibited visual growth, while those for the triazoles (ITC, VRC, PSC) were 80% inhibition compared to the growth control. Caspofungin endpoints were read as minimum effective concentrations (MECs) [24,25]. Results for AMB and CAS were 1 and 2, and 0.125 and 0.25 µg/ml, respectively. Results for the triazoles were 0.25 and 0.25, >16, and 0.06 and 0.06 µg/ml for ITC, VRC, and PSC, respectively. No defined breakpoints are currently available for these antifungal agents against this organism.

Discussion

The genus *Geosmithia* currently contains numerous species formerly classified as *Penicillium*. *Geosmithia argillacea* (Stolk, H.C. Evans & T. Nilsson) [26], was originally described as a new thermotolerant *Penicillium* species by Stolk *et al*. who isolated the type strain from a high-temperature mine waste tip in 1969 [21]. In 1979 Pitt [26] erected the genus *Geosmithia* to distinguish isolates previously known as *Penicillium* spp. but which formed conidia borne as cylinders from cylindrical, rough-walled phialides lacking narrow necks, as in *Penicillium* and *Paecilomyces*, and that produced conidia that were not typically some shade of green. In 1994, Yaguchi *et al*. [27] described a new species of *Talaromyces, T. eburneus,* from the soil in Taiwan. In a subsequent investigation [28] of an outbreak of fungal contamination of pasteurized pineapple juice in the beverage industry, he recovered a strain of *Talaromyces eburneus* having a *Geosmithia* anamorph (asexual form). As this species had not been previously regarded as thermophilic, sequence analysis was performed to compare this species with the type strain of *T. eburneus*, and 3 strains of *Geosmithia argillacea*. The D1/D2 regions of 28S rDNA for all strains were identical, thereby confirming *T. eburneus* as the teleomorph (sexual form) of *Geosmithia argillacea* [22,28]. The etiologic agent in the dog in the current report was initially thought to be a *Penicillium* species based on its microscopic morphology, however a more detailed examination of the morphologic features combined with molecular characterization confirmed the identification as *G. argillacea* and emphasizes the utility of ITS and D1/D2 sequencing. Previous reports of disseminated infection with *Penicillium* species may have suffered from similar misidentification.

To our knowledge this is the first report of a *Geosmithia* species causing disseminated disease in either humans or animals. *Geosmithia argillacea* was isolated from a pleural cavity drain from a human, though the method of determining fungal identity was not described [29]. More recently, *G. argillacea* has been considered a potential pathogen in cystic fibrosis lung disease [30]. The breed and gender of the dog and the physical manifestations of infection with *Geosmithia* in this report were typical of those associated with disseminated aspergillosis [12]. We suspect this dog may have had a predisposing immunodeficiency, though tests of immune function were not performed due to financial constraints. Antifungal therapy was not administered as prognosis for other seemingly similar disseminated mycoses such as aspergillosis and penicilliosis is so poor. *In vitro* antifungal susceptibility testing performed post-mortem suggested susceptibility to itraconazole, posaconazole, and caspofungin raising the possibility that treatment may have had a beneficial effect. The ability of these drugs to penetrate all infected tissues, however, is questionable. Amphotericin B may also have been efficacious had a liposomal

preparation been used, while voriconazole clearly lacked activity, *in vitro*.

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References

1 Bruchim Y, Elad D, Klainbart S. Disseminated aspergillosis in two dogs in Israel. *Mycoses* 2006; **49**: 130–133.
2 Dallman MJ, Dew TL, Tobias L, Doss R. Disseminated aspergillosis in a dog with diskospondylitis and neurologic deficits. *J Am Vet Med Assoc* 1992; **200**: 511–513.
3 Day MJ, Eger CE, Shaw SE, Penhale WJ. Immunologic study of systemic aspergillosis in German shepherd dogs. *Vet Immunol Immunopathol* 1985; **9**: 335–347.
4 Day MJ, Penhale WJ. Humoral immunity in disseminated *Aspergillus terreus* infection in the dog. *Vet Microbiol* 1988; **16**: 283–294.
5 Jang SS, Dorr TE, Biberstein EL, Wong A. *Aspergillus deflectus* infection in four dogs. *J Med Vet Mycol* 1986; **24**: 95–104.
6 Kabay MJ, Robinson WF, Huxtable CR, McAleer R. The pathology of disseminated *Aspergillus terreus* infection in dogs. *Vet Pathol* 1985; **22**: 540–547.
7 Kahler JS, Leach MW, Jang S, Wong A. Disseminated aspergillosis attributable to *Aspergillus deflectus* in a springer spaniel. *J Am Vet Med Assoc* 1990; **197**: 871–874.
8 Kelly SE, Shaw SE, Clark WT. Long-term survival of four dogs with disseminated *Aspergillus terreus* infection treated with itraconazole. *Aust Vet J* 1995; **72**: 311–313.
9 Mullaney TP, Levin S, Indrieri RJ. Disseminated aspergillosis in a dog. *J Am Vet Med Assoc* 1983; **182**: 516–518.
10 Robinson WF, Connole MD, King TJ, Pitt JI, Moss SM. Systemic mycosis due to *Aspergillus deflectus* in a dog. *Aust Vet J* 2000; **78**: 600–602.
11 Wood GL, Hirsh DC, Selcer RR, Rinaldi MG, Boorman GA. Disseminated aspergillosis in a dog. *J Am Vet Med Assoc* 1978; **172**(6): 704–707.
12 Schultz RM, Johnson EG, Wisner ER, *et al*. Clinicopathologic and diagnostic imaging characteristics of systemic aspergillosis in 30 dogs. *J Vet Intern Med* 2008; **22**: 851–859.
13 Zanatta R, Miniscalco B, Guarro J, *et al*. A case of disseminated mycosis in a German shepherd dog due to *Penicillium purpurogenum*. *Med Mycol* 2006; **44**: 93–97.
14 Littman MP, Goldschmidt MH. Systemic paecilomycosis in a dog. *J Am Vet Med Assoc* 1987; **191**: 445–447.
15 Gene J, Blanco JL, Cano J, Garcia ME, Guarro J. New filamentous fungus *Sagenomella chlamydospora* responsible for a disseminated infection in a dog. *J Clin Microbiol* 2003; **41**: 1722–1725.
16 Koller LD, Patton NM, Whitsett DK. Adiaspiromycosis in the lungs of a dog. *J Am Vet Med Assoc* 1976; **169**: 1316–1317.
17 Smith AN, Spencer JA, Stringfellow JS, Vygantas KR, Welch JA. Disseminated infection with *Phialemonium obovatum* in a German shepherd dog. *J Am Vet Med Assoc* 2000; **216**: 708–712.
18 White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand D, Sninsky J, White TC (eds). *PCR-Protocols and Applications – A Laboratory Manual*. New York: Academic Press, 1990: 315–322.
19 Kurtzman CP, Robnett CJ. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* 1997; **35**: 1216–1223.
20 Peterson SW, Kurtzman CP. Phylogenetic relationships among species of the genus *Issatchenkia Kudriavzev*. *Antonie Van Leeuwenhoek* 1990; **58**: 235–240.
21 Stolk AC, Evans HC, Nilsson T. *Penicillium argillaceum* sp. nov., a thermotolerant *Penicillium*. *Trans British Mycol Soc* 1969; **53**: 307–311.
22 Mouchacca J. Heat tolerant fungi and applied research: addition to the previously treated group of strictly thermotolerant species. *World J Microbiol Biotechnol* 2007; **23**: 1755–1770.
23 Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. *CLSI document M38-A*. 2002; Clinical and Laboratory Standards Institute, Wayne, PA.
24 Arikan S, Lozano-Chiu M, Paetznick V, Rex JH. *In vitro* susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob Agents Chemother* 2001; **45**: 327–330.
25 Kurtz MB, Heath IB, Marrinan J, *et al*. Morphological effect of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-beta-D-glucan synthase. *Antimicrob Agents Chemother* 1994; **38**: 1480–1489.
26 Pitt JI. *Geosmithia gen.* nov. for *Penicillium lavendulum* and related species. *Canadian J Bot* 1979; **57**: 2021–2030.
27 Yaguchi T, Someya A, Udagawa S. Two new species of *Talaromyces* from Taiwan and Japan. *Mycoscience* 1994; **35**: 249–255.
28 Yaguchi T, Edagawa S, Nishimura K. *Geosmithia argillacea* is the anamorph of *Talaromyces eburneus* as a heat resistant fungus. *Cryptogamie, Mycologie* 2005; **26**: 131–141.
29 Mycoses Newsletter. October, 1998: **7** (2). ISSN 1170-7062. [Newsletter on the internet]. Available from: http://www.mycology.adelaide.edu.au/downloads/Newsletter.pdf
30 Barton FC, Borman AM, Johnson EM, *et al*. *Geosmithia argillacea* – a potential new pathogen in cystic fibrosis lung disease? Abstracts of the 31st European Cystic Fibrosis Conference. *J Cystic Fibrosis* 2008; **7**(Suppl. 2): S50.

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Disseminated phaeohyphomycosis in weedy seadragons (*Phyllopteryx taeniolatus*) and leafy seadragons (*Phycodurus eques*) caused by species of *Exophiala*, including a novel species

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Abstract. During the period from January 2002 to March 2007, infections by melanized fungi were identified with greater frequency in aquarium-maintained leafy seadragons (*Phycodurus eques*) and weedy seadragons (*Phyllopteryx taeniolatus*), pivotal species to the educational and environmental concerns of the aquarium industry and conservation groups. The objective of this study was to characterize the pathology and identify fungi associated with phaeohyphomycotic lesions in these species. Samples from 14 weedy and 6 leafy seadragons were received from 2 institutions and included fresh, frozen, and formalin-fixed tissues from necropsy and biopsy specimens. Fresh and frozen tissues were cultured for fungi on Sabouraud dextrose agar only or both Sabouraud dextrose agar and inhibitory mold agar with gentamicin and chloramphenicol at 30°C. Isolates were processed for morphologic identification and molecular sequence analysis of the internal transcribed spacer region and D1/D2 domains of the large subunit ribosomal RNA gene. Lesions were extensive and consisted of parenchymal and vascular necrosis with fungal invasion of gill (11/20), kidney (14/20), and other coelomic viscera with or without cutaneous ulceration (13/20). *Exophiala* sp. isolates were obtained from 4 weedy and 3 leafy seadragons and were identified to species level in 6 of 7 instances, namely *Exophiala angulospora* (1) and a novel species of *Exophiala* (5), based on nucleotide sequence comparisons and phylogenetic analyses. Disseminated phaeohyphomycosis represents an important pathologic condition of both weedy and leafy seadragons for which 2 species of *Exophiala*, 1 a novel species, have been isolated.

Key words: *Exophiala*; phaeohyphomycosis; *Phycodurus eques*; *Phyllopteryx taeniolatus*; seadragons.

Introduction

The term ''phaeohyphomycosis'' is collectively used for cutaneous, subcutaneous, and systemic diseases caused by several genera of septate dark-walled fungi, referred to as ''dematiaceous,'' ''phaeoid,'' or ''melanized.'' Melanized fungi exhibit a high degree of molecular diversity with more than 100 species in 60 genera.[40] These agents, classified in various orders of the fungal kingdom, are ubiquitous and primarily recognized as soil saprophytes, plant pathogens, and environmental contaminants. Melanized fungi have been associated with disease in humans,[6,57] mammals,[11,18,28,61] birds,[32,56] amphibians,[12] reptiles,[30] fish,[19,60] and invertebrates.[8,62]

Of special interest are members of the order *Chaetothyriales*, such as *Exophiala* and *Cladophialophora*, which are ecologically different and seem to be associated with assimilation of alkylbenzenes, compounds that are also present in vertebrate bodies.[50] These fungi are regularly encountered as causative agents of mycoses of medical and veterinary importance; only *Onygenales*, the order containing dermatophytes and dimorphic pathogens, has a comparable number of clinically relevant fungi.[16,17]

Species of *Exophiala* represent a source of emerging fungal cutaneous, subcutaneous, and systemic infections, especially in immunocompromised human patients,[9,36,52,53,68] and animals.[10,27,31,39] In teleostean and cartilaginous fishes, *Exophiala* sp. infection has been reported in Atlantic salmon (*Salmo salar* L.)[46,54] and cutthroat trout (*Oncorhynchus clarkii*),[13] caused by *Exophiala salmonis*, in channel catfish (*Ictalurus punctatus*)[42] and smooth dogfish (*Mustelus canis*)[23] due to *Exophiala pisciphila*, and in captured King George whiting (*Sillaginodes punctata*)[51] in associa-

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tion with an *Exophiala* sp. Most recently, a novel species of *Exophiala* was isolated from Japanese flounder (*Paralichthys olivaceus*).[33]

Seadragons are marine fish of the family *Syngnathidae*, which include the fused jawed fishes, such as seahorses and pipefish. Seadragons inhabit the shallow, temperate waters along the southern and western Australian coastline and Tasmania and are listed as near threatened on the Red List of Threatened Species by the International Union for the Conservation of Nature and Natural Resources (www.iucn.org). There are 2 main genera with a single species in each genus, namely the weedy seadragon (*Phyllopteryx taeniolatus*) and the leafy seadragon (*Phycodurus eques*). Seadragons are significant exhibit species of the aquarium industry and conservation groups because of their importance to research and educational efforts focused on marine coastal habitat conservation and ecosystem sustainability (www.dragonsearch.asn.au).[14,15] Over a period of 5 years from January 2002 to March 2007, slightly more than 400 seahorses, pipefish, and seadragons from several zoos and approximately a dozen commercial aquariums have been submitted to the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL; University of Connecticut, Storrs, CT) for necropsy. Of these syngnathid submissions, infections with melanized fungi were prevalent in leafy and weedy seadragons from 2 different aquariums. This report describes the characteristics of disseminated phaeohyphomycosis in seadragons caused by *Exophiala* spp., including infection by a novel (i.e., as yet undescribed) species referred to as "*Exophiala* sp. nov."

Material and methods

Animals

Specimens consisted of juvenile and adult, captive-hatched seadragons reared in Australia prior to legal importation into the United States. Gender was undetermined for most animals due to sexual monomorphism when not in breeding condition. Animals were housed in groups of 1–10 animals in both species-specific and mixed species groups. Animals were maintained in filtered, natural, or artificial sea water at temperatures of 14–16°C, pH 8.0–8.2, total ammonia nitrogen <0.07 mg/l, nitrite 0 mg/l, nitrate <30 mg/l, and salinity 29–36 g/l in tanks ranging from 1,000 to 8,000 liters. Life support systems included mechanical and biological filtration with protein skimmers and ultraviolet and ozone disinfection. Diet consisted of live and frozen mysis shrimp (*Mysidacea* sp.), frozen zooplankton, and live brine shrimp (*Artemia salina*).

Weedy and leafy seadragons were submitted as part of routine diagnostic investigations to the CVMDL between January 2002 and March 2007. Fish were submitted from 2 different commercial aquariums located in 2 U.S. states and were presented either live or fixed in formalin. Seadragons were euthanized using an approximate dose of 400 mg/l of tricaine methanesulfonate[a] and were observed for 15–30 min past the last active opercular movement in consideration of the guidelines provided by the American Veterinary Medical Association[3] at the CVMDL or at the submitting institutions, or the animals died naturally. After initial identification of *Exophiala* sp., some animals were treated with little success using a variety of topical and systemic antifungal agents, including fluconazole,[b] voriconazole,[b] itraconazole,[c] terbenifine,[d] 37% w/v formaldehyde solution,[d] methylene blue,[d] malachite green,[e] acriflavine,[e] and Virkon.[f] Therapy for concurrent bacterial and protozoal infections varied for each case and included at least 1 of the following drugs: ceftazidime,[g] oxytetracycline, triple sulfa powder, metronidazole, kanamycin sulfate powder,[e] and chloroquine.[h]

Necropsy and histopathology

Gross necropsies were performed within 12 hr of death. Representative tissue samples or swabs of lesions were aseptically collected for wet mount preparations and microbial culture. Animals were dissected into multiple tissue samples or had gills and coelomic viscera exposed by removal of the operculums and a ventral midline incision with or without removal of the lateral body wall. Tissue samples or partially opened whole specimens were then fixed by immersion in 10% neutral buffered formalin. Bony tissues were decalcified after fixation using 0.5 M (molar [solution]) ethylenediamine tetra-acetic acid for 24–36 hr prior to trimming.

For preparation of histologic sections, formalin-fixed tissue samples were trimmed to fit plastic cassettes, routinely processed, embedded in paraffin, sectioned at 4 μm, mounted on glass slides, stained with hematoxylin and eosin, and then examined by bright field microscopy. Additional sections were stained with Fontana-Masson (FM), periodic acid–Schiff (modified McManus stain), and Grocott's methenamine silver techniques to highlight histomorphologic and staining characteristics of the fungi.[5,59]

Microbial culture and fungus identification

Tissue samples and swabs were submitted for microbial testing, which included aerobic bacterial culture and separate fungal culture. Bacterial cultures from 4 weedy seadragons and 1 leafy seadragon were performed at a commercial veterinary diagnostic laboratory (IDEXX Laboratories, North Grafton, MA). Fungal cultures were performed at the CVMDL, where tissue samples were seared, sliced with a sterile surgical blade, and sampled with a cotton-tipped swab, which was then used to streak plates of Sabouraud dextrose agar[i] and inhibitory mold agar with gentamicin and chloramphenicol[j] in duplicate; plates were sealed using parafilm and incubated at 30°C. Swabs submitted for fungal culture were used directly to streak duplicate plates of the 2 types of media, which were then incubated at 30°C. Plates were incubated for up to 4 weeks and examined weekly for growth of mold. In cases in which

an olivaceous to black, velvety mold was isolated, 1 plate of the pair was transferred to the Fungus Testing Laboratory (The University of Texas Health Science Center at San Antonio [UTHSCSA], San Antonio, TX) for morphologic and molecular identification.

Isolates were then transferred onto potato flakes agar (PFA) plates prepared in house and incubated at 25°C.[55] Both the macroscopic morphology of the colonies and the diagnostic microscopic features were determined from this medium after approximately 12 days of incubation. Microscopic features were studied using the slide culture technique[26] and modified to contain PFA rather than water agar for both the nutrient and the moisture source. Temperature studies were performed at 30°C, 35°C, and 40°C on PFA slants; nitrate assimilation was assessed using previously described methods.[49] Brown pigment formation was evaluated on Sabouraud dextrose agar.

Molecular fungus identification and phylogenetic analysis

Genomic DNA was extracted from conidia recovered from a 72-hr PFA plate using Prepman Ultra reagent[k] according to the manufacturer's instructions. Two polymerase chain reaction (PCR) amplifications were performed on each isolate, and molecular analyses were focused on ribosomal genes for phylogenetic inference. The first reaction amplified the internal transcribed spacer (ITS) region located between the 18S and 28S rRNA genes using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[21,65] The second reaction amplified the D1/D2 region of the 28S rRNA gene using primers NL-1 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCC-GTGTTTCAAGACGG-3′).[34,48] Both PCR amplifications were performed in 50-µl reaction volumes using Triple Master Taq DNA polymerase,[d] each deoxyribonucleotide triphosphate (dNTP),[l] and primers (prepared at the UTHSCSA Nuclear Core Facility) at concentrations specified by the manufacturer's instructions. All PCR amplifications were performed in a commercial thermocycler[m] using a preprogrammed 3-step protocol as the standard program for all reactions. Cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 30 cycles, each cycle consisting of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Amplification success was confirmed by agarose gel electrophoresis. The remaining template DNA was then cleaned by polyethylene glycol precipitation.[l] Sequencing was performed on both strands using the PCR primers as sequencing primers at the UTHSCSA Advanced Nucleic Acids Core Facility. The resultant sequences were compared with available sequences at the National Center for Biotechnology Information using BLASTn (nucleotide database using a nucleotide query, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) searches of the databases,[1,2] as well as by comparison to the sequence database of black molds at the Centraalbureau voor Schimmelcultures (CBS; http://www.cbs.knaw.nl/databases/). The ITS sequences were aligned using BioNumerics 4.6 software.[n] A substitution model was calculated using MrAIC (http://www.abc.se/~nylander), and the tree was built using TREEFINDER algorithm (http://www.treefinder.de) version June 2007 and bootstrapping with 1,000 replicates; values >80 are shown with the branches.

Results

Animals

Twenty aquarium-held weedy (14) and leafy (6) seadragons were evaluated. Animals were in the collection for 2 weeks to 4 years (mean = 32 weeks) prior to onset of clinical signs. Duration of clinical signs ranged from 1 week to 6 months (mean = 8 weeks). Clinical signs included weakness, loss of appetite, lethargy, increased respiratory rate and effort, abnormal buoyancy, listing, piping at the surface of the water, and death. Fungal dermatitis was diagnosed antemortem in 6 cases via cytology or biopsy of lesions, and antemortem fungal culture isolated *Exophiala* sp. nov. in 2 such cases.

Pathologic findings

Seadragons were submitted to the CVMDL with either antemortem evidence of fungal infection from cytology or biopsy or black lesions suggestive of phaeohyphomycosis. Lesions were identified in skeletal muscle (18/20), skin (16/20), kidney (14/20), gill (11/20), swim bladder (7/20), heart (2/20), liver (3/20), spleen (1/20), muscle coats and serosa of the intestine (2/20), mesentery (1/20), and extradural sinus and spinal cord (6/20); 15 seadragons had lesions in 3 or more of these tissues (Table 1). The most obvious gross lesions were identified in the skin and consisted of 1 to several well-demarcated, occasionally extensive, ulcerations, often with raised black margins, located randomly over the head, trunk, dorsum of the tail, at the base of fins, or in skin around the cloaca (Fig. 1). Microscopically, cutaneous lesions were characterized by ulcerations of the epidermis and extensive mats of fungal hyphae that invaded dermal fibrous connective tissue and extended into the underlying hypodermis, fascia, and skeletal muscle with myonecrosis and mild histiocytic infiltrates.

Multiple, well-demarcated, and occasionally extensive black foci were identified grossly in the kidney, gill, swim bladder, and intestinal wall of seadragons upon internal examination. Microscopically, renal lesions consisted of extensive regions of necrosis involving tubules, hematopoietic interstitium, and sinusoids that were infiltrated throughout by fungal hyphae and corresponded to grossly visible black, friable parenchyma (Fig. 2). Hyphae invaded the overlying epaxial muscle with myonecrosis and mild histiocytic infiltrates. Gill lesions consisted of focally extensive necrosis of filaments, their lamellae, the

Table 1. Anatomic distribution of phaeohyphomycotic lesions in seadragons in the current study.*

Animal ID/species	Gender	Aquarium	Gill	Skin	Skeletal muscle	Kidney	Swim bladder	Heart	Intestine	Mesentery	Liver	Spleen	Extradural sinus and spinal cord†
2002 #1 LSD	F	NEAq	X	X	X								
2003 #1 WSD	U	NEAq		X									
2003 #2 WSD	M	NEAq	X	X	X	X	X						
2003 #3 LSD	U	NEAq		X	X								
2003 #4 LSD	F	NEAq	X		X	X							X
2003 #5 WSD	U	NEAq		X	X	X							
2003 #6 LSD	F	NEAq			X	X	X						
2004 #1 WSD	F	AAq	X	X	X	X	X			X	X	X	
2004 #2 WSD	U	AAq		X	X	X	X						X†
2004 #3 LSD	U	NEAq	X	X	X	X			X		X		
2004 #4 WSD	U	AAq		X	X								
2005 #1 WSD	U	NEAq	X			X							
2005 #2 WSD	U	NEAq	X	X	X	X							
2005 #3 WSD	F	AAq	X		X	X	X				X		
2005 #4 WSD	U	NEAq	X	X	X	X		X					
2005 #5 WSD	U	NEAq		X	X	X	X	X	X				X†
2006 #1 WSD	M	AAq	X	X	X	X							X
2006 #2 WSD	U	NEAq	X	X	X	X	X						X†
2006 #3 WSD	F	NEAq		X	X								X
2007 #1 LSD	F	NEAq		X	X								
Total	20		11	16	18	14	7	2	2	1	3	1	6

* LSD = leafy seadragon (*Phycodurus eques*); WSD = weedy seadragon (*Phyllopteryx taeniolatus*); M = male; F = female; U = undetermined; NEAq = New England Aquarium, Boston, MA; AAq = Adventure Aquarium, Camden, NJ.

† Fungal hyphae were present in the spinal cord.

underlying connective tissue, and blood vessels of the gill arch, with minimal to occasionally moderate loosely organized infiltrates of macrophages (Fig. 3). Lesions in the intestine and swim bladder were limited to the muscle coats and serosa and were characterized by foci of myonecrosis, infiltrated by fungal hyphae, and minimal to moderate, loosely organized infiltrates of macrophages. In the liver, hyphae invaded venules and sinusoids with dissociation and necrosis of hepatocytes and pancreatic acini in 2 instances. Hyphae coursed through venules of the liver and the reticuloendothelial stroma of the spleen and invaded mesenteric blood vessels in 1 specimen. Small numbers of hyphae were present in the extradural sinus of 6 specimens and invaded the spinal cord in 3 of these instances.

Lumina of blood vessels of gill and viscera contained intertwined fungal hyphae together with variable amounts of fibrin, serum protein, and necrotic leukocytes, accompanied by necrosis of vessel walls (Fig. 4). Fungal hyphae were 2–3 μm in width, slender, filamentous, and septate, with right-angle branching and thin parallel walls that stained brown in routine hematoxylin and eosin–stained sections and in sections prepared using the Fontana-Masson technique, consistent with the expected histochemical staining reaction of melanized fungi (Fig. 5).[66,67] Ciliated protozoa, consistent with *Uronema* sp., and aggregates of Gram-negative, rod-shaped bacteria were identified on cytology of affected gill and skin in 5 cases. Other pathologic findings included enteric coccidiosis in weedy seadragons (2/20), biliary (1/20) and renal myxozoanosis (5/20) in weedy seadragons, and parasitic (3/20) and mycobacterial (1/20) granulomas in visceral organs.

Microbial culture and fungus identification

Fungal cultures were attempted in 4 weedy and 3 leafy seadragons involving 1 or more samples from kidney, skin, liver, and/or spleen; isolates were identified to species level in 6 of 7 instances (Table 2). Isolates grew on both Sabouraud dextrose agar and inhibitory mold agar with gentamicin and chloramphenicol at 30°C over a period of 1–3 weeks. Isolates yielded velvety, olivaceous, or brown-black molds and were referred to the Fungus Testing Laboratory over several months. In most isolates, the colonies were generally restricted, were somewhat moist initially, had a moderate growth rate, were olivaceous to olivaceous-gray with a black reverse, and became velvety at maturity. Both *E. angulospora* and *Exophiala* sp. nov. were initially yeast-like but displayed budding cells throughout their growth cycle. These cells soon germinated with the formation of pale, olivaceous torulose hyphae. Conidiation was annellidic, and annelloconidia were either single-celled (2–3 μm × 4–5 μm for *Exophiala* sp. nov.; Fig. 6) or commonly angular (2–3 μm × 4–6 μm for

E. angulospora). Conidia were borne in slimy masses at the tips of flask-shaped to cylindrical annellides and from intercalary conidiogenous loci. All isolates assimilated nitrate, none were able to grow at 35°C, and none produced a brown diffusible pigment on Sabouraud dextrose agar suggestive of *E. dermatitidis*. Given the very similar microscopic and physiologic profiles of several species of *Exophiala*, only *E. angulospora*, which produces distinct angular annelloconidia, could be identified with reasonable certainty based upon morphologic features alone. A variety of Gram-negative bacteria, including *Vibrio* sp. and *Pseudomonas* sp., were cultured from lesions. No species of bacteria was consistently isolated from these lesions.

Molecular fungus identification and phylogenetic analysis

The ITS tree was built using TREEFINDER with substitution model GTR+G according to MrAIC calculations. The same program calculated bootstrapping values that gave 100% for all branches. Isolates were found to belong to distinct species of *Exophiala*. In addition to *E. angulospora*, a second, hitherto unnamed species was encountered, clearly separate from any other taxon based on rDNA ITS sequences. Because many species of black yeasts differ by mutations rather than by indels or amplicon lengths, greater than 1% ITS sequence diversity exceeds the species level in this group. Figure 7 gives an overview of clades of nearest taxa in the order *Chaetothyriales*. Sequences dH16401, dH13448, and CBS 119918 represent isolates obtained from 1 leafy seadragon sampled in 2000 by 1 of the 2 aquariums. These sequences were initially identified as *E. pisciphila* but were subsequently found to be *Exophiala* sp. nov. when submitted to the CBS for molecular characterization along with sequences from other *Exophiala* sp. isolates obtained from seadragons and other aquatic animals. Tissue samples for histopathologic evaluation were not received from this 2000 seadragon for inclusion in the current study; nevertheless, inclusion of these sequences together with sequences of *Exophiala* isolates from other fish contributes to the distinct clade designation and host predilections that characterize *Exophiala* sp. nov. Nearly all species belonged to the black yeast genus *Exophiala* having annellidic conidiogenesis, whereas *Veronaea botryosa* with large sympodial conidiophores was found to be a member of this clade. Most species in the clade have originated from watery environments and have also been isolated from diseased fish and amphibians. In some species, strains from human origins were present and mostly associated with mild cutaneous infections. Efforts are presently under way to provide a formal description of *Exophiala* sp. nov. in a taxonomic paper, wherein this species will be introduced as a novel taxon (M. J. Harrak, G. S. de Hoog, unpublished data).

Discussion

Environmental fungi are increasingly important sources of infection to humans and animals. The emergence or resurgence of fungi as pathogens, including those previously considered environmental contaminants, have been associated with a wide range of globally relevant medical, societal, and economic factors, such as increasing populations of immunocompromised individuals,[4,22,36,52,53] international travel, changes in land use and agriculture, and even migration of clouds of desert dust in the atmosphere.[25] International commerce of farmed North American bullfrogs (*Rana catesbeiana*) used for the restaurant trade, for example, has been implicated as a cause of chytridiomycosis in wild amphibians elsewhere around the globe.[41] Ornamental fish and the aquarium industry represent a commercial source of regional and international translocation of innumerable species that can afford environmental fungi the opportunity to infect new hosts.

Exophiala spp. are ubiquitous in soil and aquatic environments and often considered environmental contaminants. Reports of *Exophiala* spp. infection in domestic and wild animals are few and include subcutaneous[10] and systemic[27] lesions in cats by *E. jeanselmei* and a subcutaneous mass in the right neck of a dog by *E. dermatitidis*.[31] In nondomestic animals, *E. jeanselmei* was isolated from a subcutaneous lesion in a free-ranging eastern box turtle,[30] and an *Exophiala* sp. with close similarity to *E. pisciphila* was isolated from systemic lesions in a Galapagos tortoise.[39] *Exophiala* spp., including *Wangiella (Exophiala) dermatitidis*, *E. jeanselmei*, *E. oligosperma*, and *E. spinifera*, are more common causes of phaeohyphomycotic lesions in immunocompromised humans and encompass ocular,[47] cutaneous, subcutaneous,[68] and occasional systemic infections, wherein a history of chronic debilitating disease, altered immune status, and/or chemotherapy was reported.[9,36,52,53] *Exophiala salmonis*, known to be a pathogen of fish,[13,46,54] has been associated with subcutaneous phaeohyphomycosis in a human patient who most likely acquired the infection from a water source.[38] This highlights the zoonotic potential of *Exophiala* spp. and the potential effect aquatic pathogens may have on human health.

Exophiala spp. have been identified as significant pathogens of cultured fish, such as cutthroat trout,[13] Atlantic salmon,[46] channel catfish,[42] and Japanese flounder,[33] resulting in localized and systemic infections with a notable variety of inflammatory

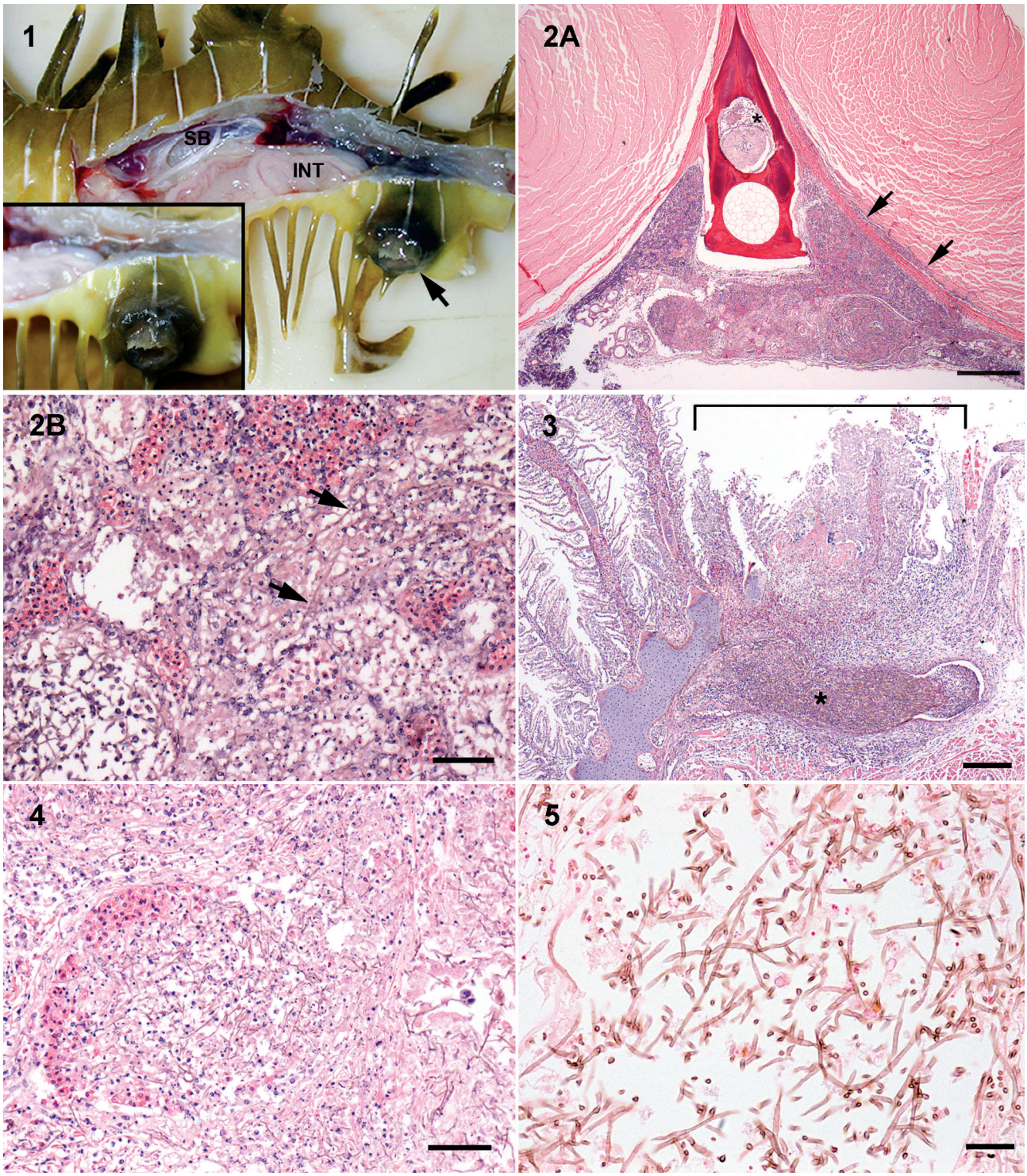

Figure 1. Skin ulcer; leafy seadragon with lateral body wall removed to expose coelomic viscera. An ulcer (arrow) is located in the skin adjacent to the cloaca. Inset: Closer view of the ulcer with raised black margins. SB = swim bladder; INT = intestine.

Figure 2. Transverse section of dorsal trunk; weedy seadragon. **A**, there is extensive necrosis involving approximately two-thirds of the renal parenchyma. Note the presence of fibrin and cells in the extradural sinus (asterisk) and an infiltrate along the fascia and margin of adjacent epaxial muscle (arrows). Hematoxylin and eosin. Bar = 500 µm. **B**, higher magnification of renal parenchyma reveals innumerable, filamentous brown fungal hyphae (arrows) coursing through necrotic tubules, interstitium, and sinusoids. Hematoxylin and eosin. Bar = 50 µm.

Figure 3. Gill; leafy seadragon. There is focally extensive necrosis of several consecutive filaments and their lamellae (bracket) overlying a region of the arch wherein a mat of densely intertwined brown fungal hyphae (asterisk) resides within the venous sinus.

Table 2. *Exophiala* sp. isolated from seadragons in the current study.*

Animal ID/Species	Aquarium	*Exophiala* sp. isolated
2002 #1 LSD	NEAq	*Exophiala* sp. nov.
2004 #3 LSD	NEAq	*Exophiala* sp. nov.
2005 #3 WSD	AAq	*Exophiala* sp. nov.
2005 #5 WSD	NEAq	*Exophiala angulospora*
2006 #1 WSD	AAq	*Exophiala* sp. nov.
2006 #3 WSD	NEAq	*Exophiala* sp. nov.

* Note the isolation of *Exophiala* sp. nov. from 2 different institutions (NEAq = New England Aquarium, Boston, MA; AAq = Adventure Aquarium, Camden, NJ). LSD = leafy seadragon (*Phycodurus eques*); WSD = weedy seadragon (*Phyllopteryx taeniolatus*).

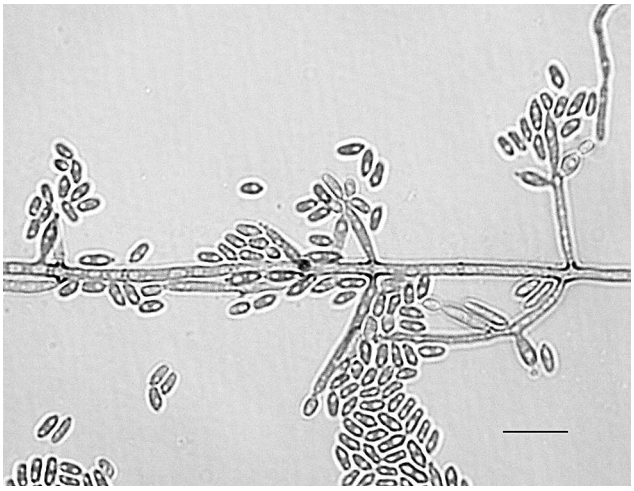

Figure 6. Microscopic colonial morphology of *Exophiala* sp. nov. showing septate hyphae with multiple annellides and conidiogenous loci bearing single-celled, approximately 2–3 μm × 4–5 μm conidia. Lactophenol cotton blue. Bar = 10 μm.

responses. A dematiaceous fungus subsequently named *Exophiala salmonis* was reported to be the cause of epizootics of cerebral mycetoma in fingerling cutthroat trout.[13] The lesion due to *E. salmonis* was characterized by granuloma formation with numerous giant cells in the brain and cranial tissues; it developed first in the brain, then extended peripherally to include surrounding cranial structures, such as the eye and gill. Similarly, in *Exophiala pisciphila* infection associated with high mortality of Atlantic salmon, hyphae invaded cranial structures, including semicircular canals, and the lateral line, accompanied by a granulomatous inflammatory reaction.[35] Contaminated food was suggested as a source of infection by *E. salmonis* in Atlantic salmon that developed granulomas in the posterior kidney.[54] *Exophiala salmonis* infection was later described in 3 Atlantic salmon held in a partial reuse system for up to 20 months.[46] Lesions in these salmon were systemic, including the kidney, and differed from earlier reports in that the host inflammatory response was predominantly granulocytic, with the formation of microabscesses. In contrast, lesions in Japanese flounder were limited to the skin.[33]

In the current study, systemic necrotizing lesions and invasion of blood vessels were consistent features of *Exophiala* spp. infection in seadragons. Necrosis was the predominant pathologic change, and the host inflammatory infiltrates were mild compared with the extent of necrosis and consisted mainly of histiocytes. Granulomas and abscesses were not consistently identified in seadragons, nor was there involvement of the brain, vestibular apparatus, or lateral line. Three of 20 seadragons did, however, have fungal hyphae that invaded the spinal cord, which may have resulted from invasion of the overlying extradural sinus.

The portal of entry in these cases is presumed to be gill or skin by traumatic inoculation or secondary inoculation of preexisting lesions, such as abrasions of the tubed snout, with subsequent hematogenous dissemination to visceral organs. Another portal of entry to consider could be direct ingestion of fungi associated with detritus and live or thawed frozen food accumulating in the tank substrate in aquarium settings. In the seadragon cases, however, there were no intestinal mucosal lesions associated with fungal invasion; instead, lesions were limited to the muscle coats and serosa, suggestive of hematogenous or transcoelomic spread.

The absence of granuloma formation or significant host inflammatory reaction to *Exophiala* spp. infection in these seadragons could be the result of an inadequate or deficient host immunologic response. No studies have been conducted to determine immune function in seadragons, but it could be that stress of captivity may be a contributory factor to reduced immune function. Fish in captive, artificial systems, or intensive rearing operations may be

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Hematoxylin and eosin. Bar = 200 μm.

Figure 4. Blood vessel, kidney; weedy seadragon. Intertwined hyphae are present in the blood vessel lumen, and there is necrosis of a segment of the wall. Hematoxylin and eosin. Bar = 200 μm.

Figure 5. Fungal hyphae, kidney; weedy seadragon. Hyphae are slender, filamentous, and septate with occasional right-angle branches. Walls of hyphae stain brown, indicative of melanin. Fontana-Masson. Bar = 25 μm.

Figure 7. Consensus tree of prevalently waterborne *Exophiala* species, based on internal transcribed spacer (ITS) ribosomal DNA of 68 strains, constructed with neighbor-joining algorithm under the HKY+G substitution model (according to MrAIC), with 1,000 bootstrap replicates (according to TREEFINDER; values ≥80 are shown with the branches) and edited with Fig Tree version 1.0. Sequences are trimmed at GGCCC to (T/C)AGGGA for comparison. *Veronaea botryosa* was selected for rooting the tree. ITS sequences from *Exophiala* sp. nov. isolates form a distinct clade separate from other *Exophiala* species and supported by very high bootstrap value (100). Symbols following taxa (*, †, ‡) indicate sequences of isolates obtained from the same individual.

immunosuppressed or otherwise compromised and therefore predisposed to infections by *Exophiala* spp. and other environmental fungi.[46] Reports of infection with pigmented fungi in tropical marine fish are usually believed to be secondary to immunosuppression resulting from transport, trauma, or confinement-induced stress.[60] There were no mortalities of other species of fish housed in the same tanks as these seadragons, suggesting that this infection is species specific. This hypothesis is supported by the fact that species in the clade of psychotolerant, waterborne melanized fungi (*Chaetothyriales*) each show somewhat different host predilections (Fig. 7).

Studies on virulence factors in phaeoid fungi, such as *Wangiella (Exophiala) dermatitidis*, have identified synthesis of melanin within cell walls as the main virulence factor.[20,29,44] Melanin scavenges free radicals and hypochlorite produced by phagocytic cells and/or binds hydrolytic enzymes, and it is also believed to be important in the formation of the fungal appressorium, a structure that aids in entering the host cells.[20,29,63] The ability of these fungi to cause disseminated infection has been associated with the resistance afforded by melanin to oxidative damage by host phagocytic mechanisms. Melanin production in the context of host infection is not limited to phaeoid fungi, however, and melanin has been identified as a virulence factor in certain dimorphic fungi, such as *Cryptococcus neoformans*,[64] *Paracoccidioides brasiliensis*,[24] and *Histoplasma capsulatum*,[45] fungi that are considered emerging pathogens in both domestic animals and humans. Few studies have examined the role of chitin as a virulence factor in melanized fungi. Chitin serves to provide additional strength to fungal cell walls,[37,63] and disruption of chitin synthases has been shown to affect growth of fungi at temperatures of infection.[37] Recently, the assimilation of alkylbenzenes, which occur as environmental pollutants but also in vertebrates as neurotransmitters, has been suggested as a virulence determinant specific to *Chaetothyriales*.[50]

Initial diagnoses in the seadragon cases in the present study were made from gross lesions observed in live and dead animals, cytologic examination of affected tissues, and characteristics of fungi in histologic sections. In instances in which frozen or fresh tissues representing suspected lesions were available, fungal culture of the kidney, skin, liver, and/or spleen consistently yielded velvety, olivaceous, or brown-black molds, which upon further molecular testing were identified as species of *Exophiala*. The genus is morphologically characterized by the presence of annellated zones producing annelloconidia from nearly undifferentiated conidiogenous cells. However, based on rDNA ITS sequence data, it was found that *Veronaea botryosa*, with large conidiophores and 2-celled sympodial conidia, is a member of this clade. Given the fact that the entire clade shows an association with watery environments (ranging from ocean water to drinking water) at cold to mild temperatures, it was concluded that ecology is a prime factor in the phylogeny of these species. Species found in somewhat warmer environments, such as bathrooms and swimming pools, are recurrently encountered as agents of mild human disease, infecting external body parts, such as skin of the extremities, and occasionally nasal sinuses (de Hoog, personal communication, 2008).

Such bacteria as *Vibrio* spp., *Pseudomonas* spp., and *Mycobacterium* spp. are commonly found in sea water and likely represent opportunistic invaders or potential members of normal skin flora.[7,43] Although a variety of Gram-negative bacteria were isolated from skin lesions, no species of bacteria was consistently isolated. Cutaneous lesions in the seadragons were often advanced when examined histologically so that a determination as to whether the inciting lesion was bacterial, fungal, or traumatic was not possible. In contrast, visceral lesions and vascular invasion were fungal in nature, and bacteria were not identified.

To the authors' knowledge, this is the first report describing disseminated phaeohyphomycosis with isolation of *Exophiala* spp. in seadragons. Seadragon habitats, such as algal covered reefs and seagrass meadows, are being adversely affected by human activities, and loss in quality and quantity of habitat has been documented (http://www.dragonsearch.asn.au; www.iucn.org).[14,15,58] Seadragons are difficult to culture and are susceptible to stress of confinement, poor diet, and trauma. Disseminated *Exophiala* spp. infection, as described in these cases, poses challenges to the management and conservation efforts of these fish.

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Sources and manufacturers

a. Finquel/MS-222, Argent Chemical Laboratories Inc., Redmond, WA.
b. Pfizer Inc., New York, NY.
c. Janssen Pharmaceutica, Beerse, Belgium.
d. Fisher Scientific Co., Pittsburgh, PA.
e. National Fish Pharmaceuticals, Tucson, AZ.
f. Virkon®, Antec International Ltd., Sudbury, Suffolk, UK.
g. GlaxoSmithKline, Brentford, Middlesex, UK.
h. Sigma-Aldrich, St. Louis, MO.
i. Northeast Laboratory Services, Waterville, ME.
j. Hardy Diagnostics, Santa Maria, CA.
k. Applied Biosystems, Foster City, CA.
l. Invitrogen Corp., Carlsbad, CA.
m. PTC-100, MJ Research, Inc., Waltham, MA.
n. Applied Maths NV, Sint-Martens-Latem, Belgium.

References

1. Altschul SF, Gish W, Miller W, et al.: 1990, Basic local alignment search tool. J Mol Biol 215:403–410.
2. Altschul SF, Madden TL, Schäffer AA, et al.: 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.
3. American Veterinary Medical Association: 2001, 2000 Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc 218:669–696.
4. Ampel NM: 1996, Emerging disease issues and fungal pathogens associated with HIV infection. Emerg Infect Dis 2:109–116.
5. Arrington JB: 1994, Bacteria, fungi and other microorganisms. *In:* Laboratory methods in histotechnology, ed. Prophet EB, Mills B, Arrington JB, Sobin LH, pp. 203–234. American Registry of Pathology, Washington, DC.
6. Barron MA, Sutton DA, Veve R, et al.: 2003, Invasive mycotic infections caused by *Chaetomium perlucidum*, a new agent of cerebral phaeohyphomycosis. J Clin Microbiol 41:5302–5307.
7. Beran V, Matlova L, Dvorska L, et al.: 2006, Distribution of mycobacteria in clinically relevant healthy ornamental fish and their aquatic environment. J Fish Dis 29:383–393.
8. Boeger WA, Pie MR, Vicente V, et al.: 2007, Histopathology of the mangrove land crab *Ucides cordatus* (Ocypodidae) affected by lethargic crab disease. Dis Aquat Organ 78:73–81.
9. Bossler AD, Richter SS, Chavez AJ, et al.: 2003, *Exophiala oligosperma* causing olecranon bursitis. J Clin Microbiol 41:4779–4782.
10. Bostock DE, Coloe PJ, Castellani A: 1982, Phaeohyphomycosis caused by *Exophiala jeanselmei* in a domestic cat. J Comp Pathol 92:479–482.
11. Bouljihad HG, Lindeman CJ, Hayden DW: 2002, Pyogranulomatous meningoencephalitis associated with a dematiaceous fungal, *Cladophialophora bantiana*, infection in a domestic cat. J Vet Diagn Invest 14:70–72.
12. Bube A, Burkhardt E, Weiß R: 1992, Spontaneous chromomycosis in the marine toad (*Bufo marinus*). J Comp Pathol 106:73–77.
13. Carmichael JW: 1966, Cerebral mycetoma of trout due to a Phialophora-like fungus. Sabouraudia 6:120–123.
14. Connolly RM, Melville AJ, Keesing JK: 2002, Abundance, movement and individual identification of leafy seadragons, *Phycodurus eques* (Pisces: Syngnathidae). Mar Freshw Res 53:777–780.
15. Connolly RM, Melville AJ, Preston KM: 2002, Patterns of movement and habitat use of leafy seadragons tracked ultrasonically. J Fish Biol 61:684–695.
16. de Hoog GS, Guarro J, Gene J, Figueras MJ, ed.: 2000, Black yeasts and their relatives. *In:* Atlas of clinical fungi, 2nd ed., pp. 374–379. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
17. de Hoog GS, Vicente V, Caligiorne RB, et al.: 2003, Species diversity and polymorphism in the *Exophiala spinifera* clade containing opportunistic black yeast-like fungi. J Clin Microbiol 41:4767–4778.
18. Elies L, Balandraud V, Boulouha L, et al.: 2003, Fatal systemic phaeohyphomycosis in a cat due to *Cladophialophora bantiana*. J Vet Med A Physiol Pathol Clin Med 50:50–53.
19. Faisal M, Elsayed E, Fitzgerald SD, et al.: 2007, Outbreaks of phaeohyphomycosis in the Chinook salmon (*Oncorhynchus tshawytscha*) caused by *Phoma herbarum*. Mycopathologia 163:41–48.
20. Feng B, Wang X, Hauser M, et al.: 2001, Molecular cloning and characterization of *WdPKS1*, a gene involved in dihydroxynaphthalene melanin biosynthesis and virulence in *Wangiella (Exophiala) dermatitidis*. Infect Immun 69:1781–1794.
21. Ferrer C, Colom F, Frasés S, et al.: 2001, Detection and identification of fungal pathogens by PCR and ITS and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol 39:2873–2879.
22. Fridkin SK: 2005, The changing faces of fungal infections in health care settings. Clin Infect Dis 41:1455–1460.
23. Gaskins JE, Cheung PJ: 1986, *Exophiala pisciphila*: a study of its development. Mycopathologia 93:173–184.
24. Gómez BL, Nosanchuk JD, Diez S, et al.: 2001, Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis* in vitro and during infection. Infect Immun 69:5760–5767.
25. Griffin DW: 2007, Atmospheric movement of microorganisms in clouds of desert dust and implications for human health. Clin Microbiol Rev 20:459–477.
26. Harris JL: 1986, Modified method for fungal slide culture. J Clin Microbiol 24:460–461.
27. Helms SR, McLeod CG: 2000, Systemic *Exophiala jeanselmei* infection in a cat. J Am Vet Med Assoc 217:1858–1861.
28. Herráez P, Rees C, Dunstan R: 2001, Invasive phaeohyphomycosis caused by *Curvularia* species in a dog. Vet Pathol 38:456–459.
29. Jacobson E: 2000, Pathogenic roles for fungal melanins. Clin Microbiol Rev 13:708–717.
30. Joyner PH, Shreve AA, Spahr J, et al.: 2006, Phaeohyphomycosis in a free-living eastern box turtle (*Terrapene carolina carolina*). J Wildl Dis 42:883–888.
31. Kano R, Kusuda M, Nakamura Y, et al.: 2000, First isolation of *Exophiala dermatitidis* from a dog: identification by molecular analysis. Vet Microbiol 76:201–205.
32. Karesh WB, Russell R, Gribble D: 1987, *Dactylaria gallopava* encephalitis in two grey-winged trumpeters, *Psophia crepitans*. Avian Dis 31:685–688.
33. Kurata O, Munchan C, Wada S, et al.: 2008, Novel *Exophiala* infection involving ulcerative skin lesions in Japanese flounder *Paralichthys olivaceus*. Fish Pathol 43:35–44.
34. Kurtzman CP, Robnett CJ: 1997, Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216–1223.
35. Langdon JS, McDonald WL: 1987, Cranial *Exophiala pisciphila* in *Salmo salar* in Australia. Bull Eur Assoc Fish Pathol 35:117–119.

36. Lau SK, Woo PC, Chiu SK, et al.: 2003, Early diagnosis of *Exophiala* CAPD peritonitis by 18S ribosomal RNA gene sequencing and its clinical significance. Diagn Microbiol Infect Dis 46:95–102.
37. Liu HS, Kauffman S, Becker JM, Szaniszlo PJ: 2004, *Wangiella* (*Exophiala*) *dermatitidis* WdChs5p, a class V chitin synthase, is essential for sustained cell growth at temperature of infection. Eukaryot Cell 3:40–51.
38. Madan V, Bisset D, Harris P, et al.: 2006, Phaeohyphomycosis caused by *Exophiala salmonis*. Br J Dermatol 154:1082–1084.
39. Manharth A, Lemberger K, Mylniczenko N, et al.: 2005, Disseminated phaeohyphomycosis due to *Exophiala* species in a Galapagos tortoise, *Geochelone nigra*. J Herpetol Med Surg 15:20–26.
40. Matsumoto T, Ajello L, Matsuda T, et al.: 1994, Developments in hyalohyphomycosis and phaeohyphomycosis. J Med Vet Mycol 32:329–349.
41. Mazzoni R, Cunningham AA, Daszak P, et al.: 2003, Emerging pathogens of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. Emerg Infect Dis 9:995–998.
42. McGinnis MR, Ajello L: 1974, A new species of *Exophiala* isolated from channel catfish. Mycopathologia 66:518–520.
43. Mylniczenko ND, Harris B, Wilborn RE, Young FA: 2007, Blood culture results from healthy and free-ranging elasmobranchs. J Aquat Anim Health 19:159–167.
44. Nosanchuk JD, Casadevall A: 2003, The contribution of melanin to microbial pathogenesis. Cell Microbiol 5:203–223.
45. Nosanchuk JD, Gomez BL, Youngchim S, et al.: 2002, *Histoplasma capsulatum* synthesizes melanin-like pigments in vitro and during mammalian infection. Infect Immun 70:5124–5131.
46. Otis EJ, Wolke RE, Blazer VS: 1985, Infection of *Exophiala salmonis* in Atlantic salmon (*Salmo salar* L). J Wildl Dis 21:61–64.
47. Patel SR, Hamersmith KM, Rupuano CJ, Cohen EJ: 2006, *Exophiala dermatitidis* keratitis after laser in situ keratomileusis. J Cataract Refract Surg 32:681–684.
48. Peterson SW, Kurtzman CP: 1990, Phylogenetic relationships among species of the genus Issatchenkia Kudriavzev. Antonie Van Leeuwenhoek 58:235–240.
49. Pincus DH, Salkin IF, Hurd NJ, et al.: 1988, Modification of potassium nitrate assimilation test for identification of clinically important yeasts. J Clin Microbiol 26:366–368.
50. Prenafeta-Boldú FX, Summerbell R, de Hoog GS: 2006, Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? FEMS Microbiol Rev 30:109–130.
51. Reuter RE, Hutchinson W, Ham J, Davis S: 2003, *Exophiala* sp. infection in captured King George whiting, *Sillaginodes punctata*. Bull Eur Assoc Fish Pathol 23:128–134.
52. Revankar SG: 2007, Dematiaceous fungi. Mycoses 50:91–101.
53. Revankar SG, Patterson JE, Sutton DA, et al.: 2002, Disseminated phaeohyphomycosis: review of an emerging mycosis. Clin Infect Dis 34:467–476.
54. Richards RH, Holliman A, Hegalson S: 1978, *Exophiala salmonis* infection in Atlantic salmon *Salmo salar* L. J Fish Dis 1:357–368.
55. Rinaldi MG: 1982, Use of potato flakes agar in clinical mycology. J Clin Microbiol 15:1159–1160.
56. Salkin IF, Dixon DM, Kemna ME, et al.: 1990, Fatal encephalitis caused by *Dactylaria constricta* var. *gallopova* in a snowy owl chick, *Nyctea scandiaca*. J Clin Microbiol 28: 2845–2847.
57. Sanchez A, Larsen R: 2007, Emerging fungal pathogens in pulmonary disease. Curr Opin Pulm Med 13:199–204.
58. Sanchez-Camara J, Booth DJ, Murdoch J, et al.: 2006, Density, habitat use and behaviour of the weedy seadragon *Phyllopteryx taeniolatus* (Teleostei: Syngnathidae) around Sydney, New South Wales, Australia. Mar Freshw Res 57:737–745.
59. Sheehan DC: 1980, Special cells and tissues. *In:* Theory and practice of histotechnology, ed. Sheehan DC, Hrapchak BB, 2nd ed., p. 277. Mosby, St. Louis, MO.
60. Silphaduang U, Hatai K, Wada S, Noga E: 2000, Cladosporiosis in a tomato clownfish (*Amphiprion frenatus*). J Zoo Wildl Med 31:259–261.
61. Singh K, Flood J, Welsh RD, et al.: 2006, Fatal systemic phaeohyphomycosis caused by *Ochroconis gallopavum* in a dog *Canis familiaris*. Vet Pathol 43:988–992.
62. Singh SM, Pathak SC, Kulkarni N, et al.: 1991, First report of phaeohyphomycosis of *Nezara viridula* Linn. (Insecta: Heteroptera) caused by *Curvularia lunata*. Mycopathologia 116:37–43.
63. Szaniszlo PJ: 2006, Virulence factors in black molds with emphasis on melanin, chitin, and *Wangiella* as a molecularly tractable model. *In:* Molecular principles of fungal pathogenesis, ed. Heitman J, Filler SG, Edwards Jr. JE, Mitchell AP, pp. 407–428. ASM Press, Washington, DC.
64. Walton FJ, Indurm A, Heltman J: 2005, Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. Mol Microbiol 57:1381–1396.
65. White TJ, Bruns T, Lee S, Taylor J: 1990, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* PCR protocols: a guide to methods and applications, ed. Innis MA, Gelfand DH, Sninsky JJ, White TJ, pp. 315–322. Academic Press, San Diego, CA.
66. Wood C, Russell-Bell B: 1983, Characterization of pigmented fungi by melanin staining. Am J Dermatopathol 5:77–81.
67. Yager JA, Scott DW: 1993, The skin and appendages. *In:* Pathology of domestic animals, ed. Jubb KVF, Kennedy PC, Palmer N, vol. 1, 4th ed., p. 668. Academic Press, San Diego, CA.
68. Zeng JS, Sutton DA, Fothergill AW, et al.: 2007, Spectrum of clinically relevant *Exophiala* species in the United States. J Clin Microbiol 45:3713–3720.

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Sequence-based identification of *Aspergillus*, *Fusarium* and *Mucorales* in the clinical mycology laboratory: Where are we and where should we go from here?

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The identification of fungal species and determination of their significance in the clinical laboratory are complex practices that help establish or exclude a fungal cause of disease. In the past, the clinical mycologist utilized a limited array of phenotypic measurements for categorizing isolates to the species level. This scenario is shifting in favour of molecular identification strategies largely due to a combination of several factors including: (i) the changing landscape of epidemiology of medically important fungi: novel organisms never before implicated in human infection are being reported from clinical samples (10, 41), (ii) reports of species-specific differences in antifungal susceptibilities of these newly recognized fungi (4, 10, 41), (iii) numerous studies demonstrating that morphology alone may not be a sufficiently objective method for species determination (7, 8, 10, 23, 41), and (iv) a growing scarcity of bench scientists and microbiologists trained in traditional mycology. With the increasing incidence of fungal infections and reports of invasive fungal infections in non-traditional populations, such as patients with critical illnesses, the onus is on the clinical microbiologist/mycologist to return a timely and accurate identification. Molecular methods are rapid with a turn around time of about 24 hours from the time of DNA extraction, yield results that are objective with data portable between labs and could be more economical in the long run.

Few topics are more controversial or evoke such a passionate response as the term "species" to a mycologist. Multiple molecular studies have demonstrated that a strategy where multiple genes (or portions there of) are sequenced and the resultant data are analyzed by phylogenetic methods is a robust strategy for fungal species recognition. This concept known as phylogenetic species recognition (PSR) (40) has been used successfully to define species in *Fusarium* and *Aspergillus* (8, 23, 29, 31,

32). The advent of PSR has greatly clarified the taxonomy of these genera and as such is a powerful tool for fungal species delimitation. However, this methodology is expensive and requires phylogenetic expertise which may be limiting factors in clinical microbiology laboratories. In reality, once a species has been delimited by PSR using several robust loci, sequence diversity within the species is known and on the basis of this knowledge, comparative sequence analyses from a single locus can be used for rapid species identification. "Cut-off scores", which are dependent on genetic diversity within and between sibling species, can be then provided.

Thus, it is important to clarify that our intent in this editorial is to address the practice of species "identification" as applied to a clinical setting and not species "classification" necessary for taxonomic categorization. Although both terms can be overlapping, the purpose of an "identification" method in a clinical microbiology laboratory is the ability to provide a specific name or epithet to an organism rapidly and with precision, without the complex experimental research or detailed phylogenetic analyses vital for a taxonomic "classification" scheme. Such specific information can then be used by the physician in a decision making algorithm that can guide patient management.

The field of medical mycology has embraced molecular methods of identification, resulting in the exploration of numerous potential targets, an explosion in the number of sequences from these loci, and recognition of previously unknown fungal species adding to the already staggering fungal diversity. On the other hand, this practice may have opened up a number of possibilities, at least from the perspective of a

mycologist in a routine microbiology laboratory, resulting in considerable uncertainty about the best possible molecular method to obtain a species identification. Realizing this, a consortium of international experts was assembled as an International Society for Human and Animal Mycology (www.isham.org) working group on fungal molecular identification. With the goal to support clinical laboratories in their efforts to identify fungal species from culture using molecular methods, the ISHAM working group agreed to begin by focusing on molecular strategies available for medically important fungi of the genera *Aspergillus*, *Fusarium* and the order *Mucorales* (Zygomycota). The advantages and limitations of these methods are discussed and the recommendations of this working group are presented in this editorial.

Comparative sequence identification strategy

Today, comparative sequence-based identification strategies can be considered the new "gold standard" for fungal species identification (39). This method is based on PCR amplification of a selected region of genomic DNA (target locus), followed by sequencing of the resulting amplicon(s). Once a consensus sequence is obtained, it can be queried against a database library and evaluation for species identification can be performed by generating dendrograms, examining percent similarity/ percent dissimilarity, or executing more sophisticated phylogenetic analyses. The current approach in clinical laboratory practice is to interpret sequence comparison results by generating a percent identity score, which is a single numeric score determined for each pair of aligned sequences and measures the number of identical nucleotide matches in relation to the length of the alignment. Cut-off scores for species identification are arbitrary and the scores can vary depending on numerous factors

75 including the quality of the sequence, the number and accuracy of existing database
76 records from the same species and locus, the length of the sequence fragment, and the
77 software program employed. At present, there is no definitive study describing an
78 absolute cut-off for same-species identity across the fungal kingdom and no
79 consensus definition exists on how to define a species using such comparative
80 sequence methodologies.
81
82 The success of a comparative sequencing strategy for the identification of a wide
83 range of clinical fungi lies mainly in the choice of the appropriate locus. The gene
84 target should be orthologous (i.e, evolved by common descent), having a high level of
85 inter-species variation combined with low levels of intra-specific variation, and
86 ideally should not undergo recombination. In addition, the target must be easy to
87 amplify and sequence using standardized "universal" primer sets. Finally, the
88 amplified DNA fragment should be within the size range obtainable with the most
89 commonly used automated DNA sequencers (~600-800 bp) and easily aligned with a
90 sequence database for comparison. Does such a utopian locus exist?
91
92 Multiple studies have demonstrated that comparative sequence-based identification
93 using the nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S rRNA and
94 ITS2) located between the nuclear small and large subunit rRNA genes (43) could be
95 employed for species complex level identification of *Aspergillus* (21) and most
96 *Mucorales* (37) and for identification within some species complexes of *Fusarium*
97 (Figure 1; 31, 44). The ITS region satisfies most of the aforementioned requirements
98 of a "universal" marker since this region can be reliably amplified for most fungi, is
99 conserved, present as multiple copies in the fungal genome, yields sufficient

taxonomic resolution for most fungi, and has the additional advantage that GenBank/ European Molecular Biology Laboratory Nucleotide Sequence Database/ DNA Data Bank of Japan (http://www.ncbi.nlm.nih.gov, http://www.ebi.ac.uk/embl/, http://www.ddbj.nig.ac.jp/) contains a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown isolate.

There is considerable consensus regarding the use of ITS sequencing as the initial step in mold identification. An international *Aspergillus* working group recently recommended the use of the ITS region for subgenus/section level identification for the genus *Aspergillus* (9). Also, the International Subcommission on Fungal Barcoding has proposed the ITS region as the prime fungal barcode or the default region for species identification (http://www.allfungi.com/its-barcode.php.). Significant disadvantages of the ITS region include: (i) insufficient hypervariability to distinguish the various species in the *Aspergillus* sections and *Fusarium* species complexes; (ii) its failure to distinguish between closely related species (sibling species) because of insufficient nucleotides differences, for example *A. lentulus* from *A. fumigatus*; and (iii) problems with the reliability of the ITS sequences deposited in the reference databases (e.g. GenBank/EMBL/DDBJ) (26).

Comparative sequence based identification strategies can be meaningful only with the availability of well-curated, robust and reliable databases that are populated with sequence data from type or reference strains (where possible), have been rigorously validated in terms of their nomenclature, and include sequences from a wide variety of target species. The most widely used database is GenBank which contains a huge number of sequences, but these are combined with unedited and non-validated

information, which may only be updated and corrected by the original submitter. Errors in fungal sequences within GenBank have been found to be as high as 20% (26). Despite calls for the process to be changed, to allow for third-party revision (11), there seems little prospect of this in the near future (34). On the other hand, smaller databases, such as those provided with commercial sequence-based identification systems, are often inadequate because of their lack of breadth (omitting many, often important species) and depth (containing few representatives of the same species) (19). To overcome these problems, specific sequence databases for particular groups of fungi, based on quality-controlled sequences have been created mainly for plant pathogenic, industrially important and ectomycorrhizal asco- and basidiomycetes fungi (e.g.: *Fusarium spp.* (FUSARIUM-ID v. 1.0 (17); http://fusarium.cbio.psu.edu/index.html), *Phaeoacremonium* spp. (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx), *Trichoderma* spp. (http://www.isth.info/morphology.php) and mycorrhizal fungi (UNITE, http://unite.ut.ee/). Two ITS databases for medical fungi are available through the CBS Fungal Biodiversity Centre and at the Westmead Millennium Institute, University of Sydney (curated database; http://www.mycologylab.org/biolomicsid.aspx) [Meyer et al. unpublished data]. Ideally the mycology community needs to find a way of combining high quality sequence and available species data present in numerous reference and research laboratories around the world.

Species identification in *Aspergillus*, *Fusarium* and the *Mucorales*

Aspergillus species

150 Taxonomically, the genus *Aspergillus* is divided into seven sub-genera, which are
151 further divided into several "sections" - for example sub-genus *Fumigati* encompasses
152 2 sections: *Fumigati* and *Cervini* (24). Clinically relevant aspergilli are represented
153 within several sections of the seven sub-genera. For instance, the medically important
154 species *A. fumigatus*, and other relatives less commonly implicated in human
155 infections such as *Neosartorya fischeri* and *A. lentulus*, fall within section *Fumigati* of
156 the genus *Aspergillus*. Given that this classification scheme is unique to the genus
157 *Aspergillus*, it is important to recognize that there can be two levels of identification:
158 (i) identification to a given species complex, e.g., discrimination of *A. fumigatus*
159 complex (subgenus *Fumigati*, section *Fumigati*) from *A. flavus* complex (subgenus
160 *Circumdati*, section *Flavi*); and (ii) identification of species within a section, e.g.,
161 discrimination of *A. fumigatus* from *A. lentulus* (both members of the section
162 *Fumigati*, subgenus *Fumigati*). Employing comparative sequence analysis of the ITS
163 region, one can rapidly and unquestionably place an *Aspergillus* isolate within the
164 respective sections, for instance *A. ustus* (Section *Usti*) or *A. terreus* (Section *Terrei*)
165 (21). In contrast, species identification within a given *Aspergillus* section, for instance
166 identification of the various species within section *Usti* (i.e *A. calidoustus*, *A. ustus*, *A.*
167 *pseudodeflectus*) can be challenging given that the ITS region has few sites that are
168 variable enough for this degree of resolution. In addition, several aspergilli have
169 overlapping morphological features rendering phenotypic identification methods
170 inadequate. Numerous studies have demonstrated that comparative sequence analyses
171 of protein coding regions such as β-tubulin, calmodulin and rodlet A can identify
172 species within sections *Fumigati*, *Usti*, *Nigri* and *Terrei* (4, 7, 8, 23, 36, 41).
173

174 Recognizing the growing role of molecular methods in *Aspergillus* species
175 identification, an international *Aspergillus* working group (9) proposed the following
176 recommendations: (i) the term "species complex" as an alternate to "section" (ii) use
177 of sequences from the ITS region for identification of *Aspergillus* isolates to the
178 species complex level; (iii) comparative sequence analyses of the β-tubulin region for
179 species identification within a complex. This recommendation can be advantageous to
180 clinical laboratories that rely on comparative sequence analyses of the ITS region
181 (which are not variable enough for species identification within a section) and/ or
182 morphology for species identification (where overlapping morphologies can hinder
183 resolution of species within the sections) as they can report the identification of an
184 unknown organism to species complex, for instance *A. terreus* complex. Thus the
185 term "complex" in such an identification scheme would indicate the placement of the
186 isolate within a species complex, but does not identify the isolate to a species within
187 the complex.
188
189 ***Fusarium* species**
190 *Fusarium* species have emerged over the past three decades as an important genus of
191 filamentous molds causing opportunistic infections in humans (27). Detailed
192 molecular studies employing sequences of multiple loci such as elongation
193 factor (EF-1 α) (17), β-tubulin (*β-TUB*), calmodulin (*CAM*), RNA polymerase II
194 second largest subunit (*RPB2*; Figure 1) and subsequent phylogenetic analyses of
195 medically important fusaria have revealed the presence of multiple cryptic species
196 within each morphologically recognized "morphospecies". For instance, '*Fusarium*
197 *solani'* actually represents a complex (i.e., *F. solani* species complex, FSSC; Figure
198 1) of over 45 phylogenetically distinct species of which at least twenty are associated

199 with human infections (31, 44). Similarly, members of the *Fusarium oxysporum*
200 species complex (FOSC) are phylogenetically diverse (31, 44), as are members of the
201 *F. incarnatum-equiseti* (FIESC) and *F. chlamydosporum* species complexes (FCSC;
202 Figure 1, 30) and O'Donnell, unpublished data). Cases involving the latter two
203 complexes are typically reported, as the polytypic morphospecies *F. incarnatum/F.*
204 *semitectum/F. equiseti* and *F. chlamydosporum* respectively (38). Available data
205 clearly demonstrate that sequences from the nuclear ribosomal internal transcribed
206 spacer region (ITS) and domains D1 and D2 of the 28S rDNA large subunit (LSU) are
207 too conserved to resolve most clinically important fusaria at the species level (31, 44),
208 despite reports to the contrary (16, 20). Moreover, use of the ITS rDNA within the
209 *Gibberella fujikuroi* species complex (GFSC) and FOSC (29), and β-tubulin within
210 the FIESC and FSSC should be avoided due to paralogous or duplicated divergent
211 alleles (32 and O'Donnell, unpublished data).
212
213 ***Mucorales***
214 Evolutionary relationships of species within the order *Mucorales* (of the Division
215 Zygomycota) have been investigated by phylogenetic analyses of nuclear ribosomal
216 18S and 28S rDNA and translation elongation factor (*EF-1α*) gene sequences and
217 have revealed that species within medically important genera such as *Absidia* and
218 *Mucor* appear to be polyphyletic (i.e.,from multiple evolutionary origins) (33, 42).
219 Indeed, a recent taxonomic revision of *Absidia* based on physiological, phylogenetic,
220 and morphological characters has been proposed (22), with re-classification of the
221 human pathogen *Absidia corymbifera* as *Mycocladus corymbiferus* in a new family.
222 Similarly, molecular and physiological data were used to distinguish two species

223 within the morphospecies *Rhizopus oryzae* (1, 2, 35), with the proposal that the
224 fumaric-malic acid producing species be named *R. delemar* (2).
225
226 Several recent studies have demonstrated the utility of comparative sequence analyses
227 of the nuclear ribosomal 28S rDNA D1/D2 domains, the ITS region, actin and partial
228 translation elongation factor (*EF-1α*) gene sequences for resolving at or near the
229 species level within the *Mucorales* (1, 2, 33, 37, 42). Analyses of intra- and inter-
230 species variability of ITS sequences from 54 isolates of *Mucorales* belonging to 16
231 different species was evaluated recently, and the results support ITS sequencing as a
232 reliable method for the accurate identification of most medically important *Mucorales*
233 to the species level (37). However, it is important to note that some closely related
234 species could not be resolved using ITS sequence data. Similarly, while ITS sequence
235 data can be used for the identification of several *Rhizopus* species (1, 25), they lack
236 sufficient variability to resolve *R. azygosporus* from *R. microsporus.* In addition the
237 D1/D2 domains of the 28S rDNA and the high-affinity iron permease 1 gene (*FTR1*)
238 appear to be useful targets for species identification, although the *FTR1* locus could
239 not resolve all of the clinically relevant species within *Rhizomucor* and *Mucor* (28).
240 Thus, it is readily apparent that sequencing of more phylogenetically informative gene
241 targets will be required for certain *Mucorales*, and that phylogenetic analyses of
242 several loci will be needed to fully assess species limits within the *Mucorales*.
243
244 **Relevance of species identification in the clinical microbiology laboratory**
245 An important issue to be considered when deciding the choice of loci and/or number
246 of loci is the relevance of identifying every unknown isolate to the smallest taxonomic
247 unit. In other words: Should a clinical microbiology laboratory strive to identify

every isolate to the species level or is it sufficient to identify isolates to the genus or species complex level. Species level identification of a fungal isolate recovered from a clinical specimen (especially from a sterile site from an immunocompromised patient) could be important given that species identification of appropriate isolates in high risk populations may reveal the etiological agent of disease, aid selection and monitoring of antifungal therapy, and support epidemiological investigations leading to effective infection control measures. On the other hand, many sporadic isolates do not represent clinically important disease and it may be wasteful to devote resources to identifying such isolates without an understanding of their role in disease.

After identifying the unknown fungal isolate to a species complex, should the laboratory go further to achieve species identification within a section or complex? This is difficult to answer – nevertheless each one of us in the clinical microbiology laboratory faces this question every time we recover a fungus from a high risk patient and/or read a manuscript describing yet another species within the complexes. The clinical significance of identifying isolates to species, for example *A. terreus* vs. *A. fumigatus* in the genus *Aspergillus,* is evident given the different susceptibilities to the antifungal drug amphotericin B; however the significance of identifying individual species within the complexes of aspergilli and fusaria, and to the species level within the *Mucorales* is not fully apparent at this time. Studies have shown species-specific differences in antifungal susceptibilities within *Aspergillus* section *Fumigati* (4) while other studies have shown little or no difference in antifungal susceptibilities of species within the sections *Usti* (41) and *Terrei* [Balajee et al, unpublished data]. Likewise, there appears to be limited species-specific differences in antifungal susceptibilities within the genus *Fusarium* (3, 6, 31). On the other hand, considerable interspecific

variation in antifungal susceptibility of the *Mucorales* to polyenes and azoles has been observed *in vitro* (14, 15) and in *in vivo* animal models of zygomycosis (18), suggesting that species identification might be clinically relevant in the future as more active antifungal agents against these organisms become available and as *in vitro* break-points are defined.

Taken together, data regarding differences in pathogenicity and *in vivo* drug susceptibilities of the various species within *Aspergillus* and *Fusarium* complex do not categorically suggest that identification within these taxa will impact clinical and therapeutic decision making, at least at the current time. However, identification to species/ strain level could inform the epidemiology of fungal infections and can be critical in outbreak investigations (12, 30). Accordingly, the decision to identify an unknown isolate to species level within a given section/ complex of these genera will be based on the need of the clients that the microbiology laboratory is serving (high risk versus low risk populations), site of isolation of the fungus (sterile versus non-sterile sites), funds and personnel available.

CLSI recommendations for fungal species identification.

At present, DNA target sequencing can provide a quantitative metric to classify fungi; however sequencing results can create laboratory uncertainty when assigning microorganisms to their appropriate taxonomical groups. Realizing this, in May 2008, the Clinical and Laboratory Standards Institute (Formerly NCCLS) published a document, "Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing" to address the challenges of sequence analyses in general clinical laboratory practice (13). Specifically, the CLSI guideline provides a systematic and

298 uniform approach to identify fungi by broad-range DNA target sequencing in the
299 clinical laboratory. The document establishes guidelines for primer design, quality
300 control parameters for amplification and sequencing, measurement of sequence
301 quality, and assessment of reference databases. Since consensus has not yet been
302 achieved in multilocus DNA sequencing, and since most clinical laboratories do not
303 have the resources to perform such analyses, the guideline focuses on the most
304 commonly used target, the ITS region. For specific taxonomic groups, tables are
305 provided to describe the relative strengths and limitations of individual DNA targets
306 and list alternative DNA targets for those laboratories pursuing further phylogenetic
307 resolution. Finally, the document discusses reporting strategies that are clinically
308 relevant for specific groups of microorganisms. Since our current understanding of the
309 diversity of clinically important phylogenetic species within *Aspergillus* and
310 *Fusarium* is in flux and the biological importance of drawing finer phylogenetic
311 distinctions remains to be determined, the guideline recommends that for certain taxa,
312 clinical laboratories report sequence results for isolates only to the level of genus or
313 species complex.
314
315 The CLSI document is largely centered around the ITS region as a target because of
316 the general applicability, research backing, and literature validation of this target.
317 Similar to CLSI, the WG details differences between species complexes and
318 individual species, and presents alternative targets that might offer the user more
319 specific species identification in *Aspergillus* and *Fusarium* if such information is
320 needed.
321
322 **Recommendations of the Working group**

If the goal is to identify an unknown organism with no a priori knowledge, then the ITS region is a reasonable and extensively used choice for species complex identification within the genera *Aspergillus*, *Fusarium* and to most species within the Mucorales.

Such a consensus on the employment of the ITS region as the default locus for use in the clinical laboratory setting would achieve international consistency in the way that other collaborative initiatives, such as the EORTC/MSG diagnostic criteria for invasive fungal infection (5), have been successful. This consensus should have the effect of enhancing the publication of ITS sequences and focusing commercial efforts on this strategy. Clinical laboratories that have been reluctant to adopt molecular technology in an atmosphere of conflicting opinions and evidence are more likely to implement methodology that has international backing. In addition, the quality of clinical and other research publications would be improved and harmonized based on the use of a universal locus.

This working group acknowledges the known shortcomings of the ITS locus and therefore recommends a staged sequence-based identification strategy (Figure 2) for identification of aspergilli, fusaria and the *Mucorales* in a clinical microbiology laboratory. Based on this proposed algorithm, when an unknown fungal isolate is received in a clinical microbiology laboratory, after initial morphological examination the laboratory can pursue morphological or molecular identification methods or choose a combination of both methods (Figure 2). When further resolution is required, comparative sequence analyses of one or several protein coding regions can

348 be performed for species level identification within *Aspergillus* and *Fusarium*
349 complex.
350
351 There is no universal agreement on the identity cut-off values that should be applied
352 for same species identity and thus a certain degree of interpretation will be required,
353 at least until the issues already discussed have been resolved. At this time, the CLSI
354 guidelines do not provide cut-off values because, at the time of writing, the available
355 data did not support such cut-off values for fungi. Nevertheless, it will be important
356 for the mycological community to refine guidelines in this difficult area for users in
357 the clinical setting, to ensure consistency of interpretation. Thus far, analysing ITS
358 sequence data from >600 *Aspergillus* isolates from three different laboratories
359 (Balajee SA, Meyer W, Velegraki A; unpublished data) and employing both "in
360 house" sequence databases and the GenBank/EMBL/DDBJ database for sequence
361 comparison, a percent identity of 94 - 100% to the respective type/validated strain is
362 proposed for species complex level identification within the genus *Aspergillus*. For
363 the genus *Fusarium* (Figure 1) and within most species within the *Mucorales*, we
364 propose that if the ITS sequence of an unknown fungal isolate yields a percent
365 identity of ≥ 99% to a type/reference strain, the isolate can be placed within one of
366 six clinically relevant species complexes. When ITS comparative sequence analyses
367 yield ambiguous data, the clinical laboratory may consider sending the isolate to a
368 reference laboratory for identification.
369
370 When performing comparative sequence analyses (as outlined in Figure 2) it is
371 imperative to understand that the percent identity scores generated using
372 GenBank/EMBL/DDBJ are influenced by numerous factors including the quality of

the sequence, the number and accuracy of existing GenBank/EMBL/DDBJ records for the same species and locus, and the completeness of the sequence (double-stranded sequence). Importantly, because outputs can be ranked by maximum score, total score, or percent identity, and searches can be customized for parameter preferences (i.e., gap penalties and BLAST algorithm), users should take advantage of some of the tutorials and background information prior to performing searches.

In order to improve the accuracy of sequence data, the working group further emphasizes the importance of completing database record fields (especially those of GenBank®/EMBL/DDBJ) correctly when submitting sequences for inclusion in these databases. The teleomorph name should be included if known and available for the organism and species names should follow guidelines established by the International Code of Botanical Nomenclature. Species identity and sequence accuracy can be confirmed with reference to other sources such as the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl), the UK National Collection of Pathogenic Fungi (http://www.hpacultures.org.uk) and Mycobank (http://www.mycobank.org.). It must be remembered however that isolates in these collections were mostly identified by morphology alone.

Comparative sequence based identification is an evolving area of research with the constant addition of new sequences at novel and traditional loci to many different databases. Future studies will be needed to assess the validity of the proposal made in this editorial and to examine its utility in the clinical setting. As noted, the CLSI document is intended to be updated periodically, so that additional research based evidence can be translated into better defined algorithms and guidelines of practical

benefit. It is an important beginning and, together with the efforts of the working group, it should help guide and inform development of this clinical mycology laboratory methodology.

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404

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408 **References**

409 1. **Abe, A., Y. Oda, K. Asano, and T. Sone.** 2006. The molecular phylogeny of
410 the genus *Rhizopus* based on rDNA sequences. Biosci. Biotechnol. Biochem.
411 **70:**2387-2393.

412 2. **Abe, A., Y. Oda, K. Asano, and T. Sone.** 2007. *Rhizopus delemar* is the
413 proper name for *Rhizopus oryzae* fumaric-malic acid producers. Mycologia.
414 **99:**714-722.

415 3. **Alastruey-Izquierdo, A., M. Cuenca-Estrella, A. Monzon, E. Mellado, and**
416 **J. L. Rodriguez-Tudela.** 2008. Antifungal susceptibility profile of clinical
417 *Fusarium* spp. isolates identified by molecular methods. J. Antimicrob.
418 Chemother. **61:**805-809.

419 4. **Alcazar-Fuoli, L., E. Mellado, A. Alastruey-Izquierdo, M. Cuenca-**
420 **Estrella, and J. L. Rodriguez-Tudela.** 2008. *Aspergillus* section *Fumigati*:
421 antifungal susceptibility patterns and sequence-based identification.
422 Antimicrob. Agents. Chemother. **52:**1244-1251.

423 5. **Ascioglu, S., J. H. Rex, B. de Pauw, J. E. Bennett, J. Bille, F. Crokaert, D.**
424 **W. Denning, J. P. Donnelly, J. E. Edwards, Z. Erjavec, D. Fiere, O.**
425 **Lortholary, J. Maertens, J. F. Meis, T. F. Patterson, J. Ritter, D. Selleslag,**
426 **P. M. Shah, D. A. Stevens, and T. J. Walsh.** 2002. Defining opportunistic
427 invasive fungal infections in immunocompromised patients with cancer and
428 hematopoietic stem cell transplants: an international consensus. Clin. Infect.
429 Dis. **34:**7-14.

430 6. **Azor, M., J. Gene, J. Cano, D. A. Sutton, A. W. Fothergill, M. G. Rinaldi,**
431 **and J. Guarro.** 2008. In vitro antifungal susceptibility and molecular
432 characterization of clinical isolates of *Fusarium verticillioides* (*F.*

moniliforme) and *Fusarium thapsinum*. Antimicrob. Agents. Chemother. **52:**2228-2231.

7. **Balajee, S. A., J. Gribskov, M. Brandt, J. Ito, A. Fothergill, and K. A. Marr.** 2005. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. J. Clin. Microbiol. **43:**5996-5999.

8. **Balajee, S. A., J. L. Gribskov, E. Hanley, D. Nickle, and K. A. Marr.** 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot. Cell **4:**625-632.

9. **Balajee, S. A., J. Houbraken, P.E.Verweij, S.B. Hong, T. Yaghuchi, J.Varga and R.A.Samson.** 2007. *Aspergillus* species identification in the clinical setting. Stud.Mycol. **59:**39-46.

10. **Balajee, S. A., M. D. Lindsley, N. Iqbal, J. Ito, P. G. Pappas, and M. E. Brandt.** 2007. Nonsporulating clinical isolate identified as *Petromyces alliaceus* (Anamorph *Aspergillus alliaceus*) by morphological and sequence-Based Methods. J. Clin. Microbiol. **45:**2701-2703.

11. **Bidartondo, M. I.** 2008. Preserving accuracy in GenBank. Science **319:**1616.

12. **Chang, D. C., G. B. Grant, K. O'Donnell, K. A. Wannemuehler, J. Noble-Wang, C. Y. Rao, L. M. Jacobson, C. S. Crowell, R. S. Sneed, F. M. Lewis, J. K. Schaffzin, M. A. Kainer, C. A. Genese, E. C. Alfonso, D. B. Jones, A. Srinivasan, S. K. Fridkin, and B. J. Park.** 2006. Multistate outbreak of *Fusarium* keratitis associated with use of a contact lens solution. JAMA. **296:**953-963.

13. **Clinical and Laboratory Standards Institute.** 2008. Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing: Guideline. CLSI document MM18-A.

14. **Dannaoui, E., J. F. Meis, J. W. Mouton, and P. E. Verweij.** 2002. In vitro susceptibilities of *Zygomycota* to polyenes. J. Antimicrob. Chemother. **49:**741-744.

15. **Dannaoui, E., J. Meletiadis, J. W. Mouton, J. F. Meis, and P. E. Verweij.** 2003. In vitro susceptibilities of zygomycetes to conventional and new antifungals. J. Antimicrob. Chemother. **51:**45-52.

16. **Ferrer, C., J. Alio, A. Rodriguez, M. Andreu, and F. Colom.** 2005. Endophthalmitis caused by *Fusarium proliferatum*. J. Clin. Microbiol. **43:**5372-5375.

17. **Geiser, D. M., M. D. Jimenez-Gasco, S. C. Kang, I. Makalowska, N. Veeraraghavan, T. J. Ward, N. Zhang, G. A. Kuldau, and K. O'Donnell.** 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. Eur. J. Plant Pathol.**110:**473-479.

18. **Greenberg, R. N., L. J. Scott, H. H. Vaughn, and J. A. Ribes.** 2004. Zygomycosis (mucormycosis): emerging clinical importance and new treatments. Curr. Opin. Infect. Dis. **17:**517-525.

19. **Hall, L., S. Wohlfiel, and G. D. Roberts.** 2004. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. J. Clin. Microbiol. **42:**622-626.

20. **Hennequin, C., E. Abachin, F. Symoens, V. Lavarde, G. Reboux, N. Nolard, and P. Berche.** 1999. Identification of *Fusarium* species involved in human infections by 28S rRNA gene sequencing. J. Clin. Microbiol. **37:**3586-3589.

21. **Hinrikson, H. P., S. F. Hurst, T. J. Lott, D. W. Warnock, and C. J. Morrison.** 2005. Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. J. Clin. Microbiol. **43:**2092-2103.

22. **Hoffmann, K., S. Discher, and K. Voigt.** 2007. Revision of the genus *Absidia* (*Mucorales*, *Zygomycetes*) based on physiological, phylogenetic, and morphological characters; thermotolerant *Absidia* spp. form a coherent group, *Mycocladiaceae* fam. nov. Mycol. Res. **111:**1169-1183.

23. **Hong, S. B., S. J. Go, H. D. Shin, J. C. Frisvad, and R. A. Samson.** 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia **97:**1316-1329.

24. **Klich, M. A.** 2006. Identification of clinically relevant aspergilli. Med. Mycol. **44 Suppl:**127-131.

25. **Nagao, K., T. Ota, A. Tanikawa, Y. Takae, T. Mori, S. Udagawa, and T. Nishikawa.** 2005. Genetic identification and detection of human pathogenic *Rhizopus* species, a major mucormycosis agent, by multiplex PCR based on internal transcribed spacer region of rRNA gene. J. Dermatol. Sci. **39:**23-31.

26. **Nilsson, R. H., M. Ryberg, E. Kristiansson, K. Abarenkov, K. H. Larsson, and U. Koljalg.** 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS ONE **1:**e59.

27. **Nucci, M., and E. Anaissie.** 2002. Cutaneous infection by *Fusarium* species in healthy and immunocompromised hosts: implications for diagnosis and management. Clin. Infect. Dis. **35:**909-920.

28. **Nyilasi, I., T. Papp, A. Csernetics, K. Krizsan, E. Nagy, and C. Vagvolgyi.** 2008. High-affinity iron permease (FTR1) gene sequence-based molecular identification of clinically important *Zygomycetes*. Clin. Microbiol. Infect. **14:**393-397.

29. **O'Donnell, K., E. Cigelnik, and H. I. Nirenberg.** 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia **90:**465-493.

30. **O'Donnell, K., B. A. Sarver, M. Brandt, D. C. Chang, J. Noble-Wang, B. J. Park, D. A. Sutton, L. Benjamin, M. Lindsley, A. Padhye, D. M. Geiser, and T. J. Ward.** 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the 2005-06 multistate contact lens-associated U.S. keratitis outbreaks. J. Clin. Microbiol.**45**: 2235 - 2248.

31. **O'Donnell, K., D. A. Sutton, A. Fothergill, D. McCarthy, M. G. Rinaldi, M. E. Brandt, N. Zhang, and D. M. Geiser.** 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. **46:**2477-2490.

32. **O'Donnell, K.** 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia **92:**919-938.

33. **O'Donnell, K., F. Lutzoni, T. J. Ward, and G. L. Benny.** 2001. Evolutionary relationships among mucoralean fungi (*Zygomycota*): Evidence for family polyphyly on a large scale. Mycologia **93:**286-296.

34. **Pennisi, E.** 2008. DNA data. Proposal to 'Wikify' GenBank meets stiff resistance. Science **319:**1598-1599.

531 35. **Saito, K., A. Saito, M. Ohnishi, and Y. Oda.** 2004. Genetic diversity in
532 *Rhizopus oryzae* strains as revealed by the sequence of lactate dehydrogenase
533 genes. Arch. Microbiol. **182:**30-36.
534 36. **Samson, R. A., S. Hong, S. W. Peterson, J. C. Frisvad, and J. Varga.**
535 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its
536 teleomorph Neosartorya. Stud. Mycol. **59:**147–203.
537 37. **Schwarz, P., S. Bretagne, J. C. Gantier, D. Garcia-Hermoso, O.**
538 **Lortholary, F. Dromer, and E. Dannaoui.** 2006. Molecular identification of
539 zygomycetes from culture and experimentally infected tissues. J. Clin.
540 Microbiol. **44:**340-349.
541 38. **Summerbell, R. C.** 2003. *Aspergillus*, *Fusarium*, *Sporothrix*, *Piedraia*, and
542 their relatives, p. 237-498. *In* D. H. Howard (ed.), Pathogenic fungi in humans
543 and animals. Marcel Dekker, Inc., New York, N.Y.
544 39. **Summerbell, R. C., C. A. Levesque, K. A. Seifert, M. Bovers, J. W. Fell,**
545 **M. R. Diaz, T. Boekhout, G. S. de Hoog, J. Stalpers, and P. W. Crous.**
546 2005. Microcoding: the second step in DNA barcoding. Philos Trans. R. Soc.
547 Lond. B. Biol. Sci. **360:**1897-1903.
548 40. **Taylor, J. W., D. J. Jacobson, S. Kroken, T. Kasuga, D. M. Geiser, D. S.**
549 **Hibbett, and M. C. Fisher.** 2000. Phylogenetic species recognition and
550 species concepts in fungi. Fungal Genet and Biol. **31:**21-32.
551 41. **Varga, J., J. Houbraken, H. A. Van Der Lee, P. E. Verweij, and R. A.**
552 **Samson.** 2008. *Aspergillus calidoustus* sp. nov., causative agent of human
553 infections previously assigned to *Aspergillus ustus*. Eukaryot. Cell **7:**630-638.

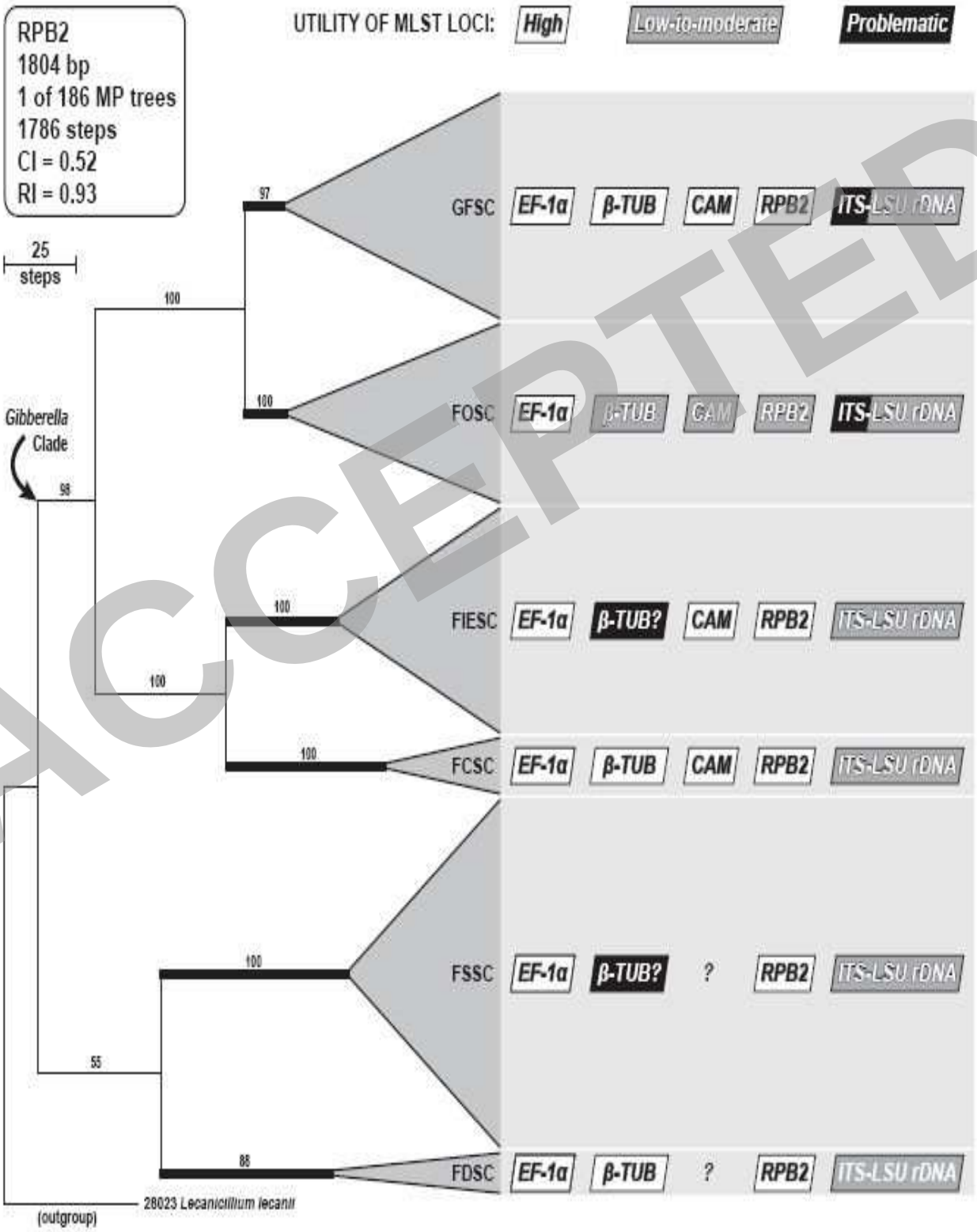
554 42. **Voigt, K., E. Cigelnik, and K. O'Donnell.** 1999. Phylogeny and PCR
555 identification of clinically important *Zygomycetes* based on nuclear ribosomal-
556 DNA sequence data. J. Clin. Microbiol. **37:**3957-3964.
557 43. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct
558 sequencing of fungal ribosomal RNA genes for phylogenetics. 315-322. *In* M.
559 A. Innis, D. H.Gelfand, J. J.Sninsky, and T. J. White (ed) . PCR protocols: a
560 guide to methods and applications. Academic Press, San Diego, CA.
561 44. **Zhang, N., K. O'Donnell, D. A. Sutton, F. A. Nalim, R. C. Summerbell, A.**
562 **A. Padhye, and D. M. Geiser.** 2006. Members of the *Fusarium solani* species
563 complex that cause infections in both humans and plants are common in the
564 environment. J. Clin. Microbiol. **44:**2186-2190.
565

Figure Legend

Figure 1. RNA polymerase II second largest subunit (*RPB2*) phylogeny of *Fusarium* (modified from Figure 1 in ref 30), showing the utility of DNA sequence data from various loci for resolving at or near the species level within six medically important species complexes. GFSC, *Gibberella fujikuroi* species complex; FOSC, *Fusarium oxysporum* species complex; FIESC, *Fusarium incarnatum-equiseti* species complex; FCSC, *Fusarium chlamydosporum* species complex; FSSC, *Fusarium solani* species complex; FDSC, *Fusarium dimerum* species complex. Loci include: *EF-1α*, translation elongation factor; *β-TUB*, beta-tubulin; *CAM*, calmodulin. Numbers above the internodes represent the frequency (%) with which they were recovered from 1,000 bootstrap replicates of the data. A sequence of *Lecanicillium lecanii* was used to root the phylogeny.

Figure 2. An algorithm for identification of an unknown filamentous fungal species in a clinical microbiology laboratory.

Figure 1



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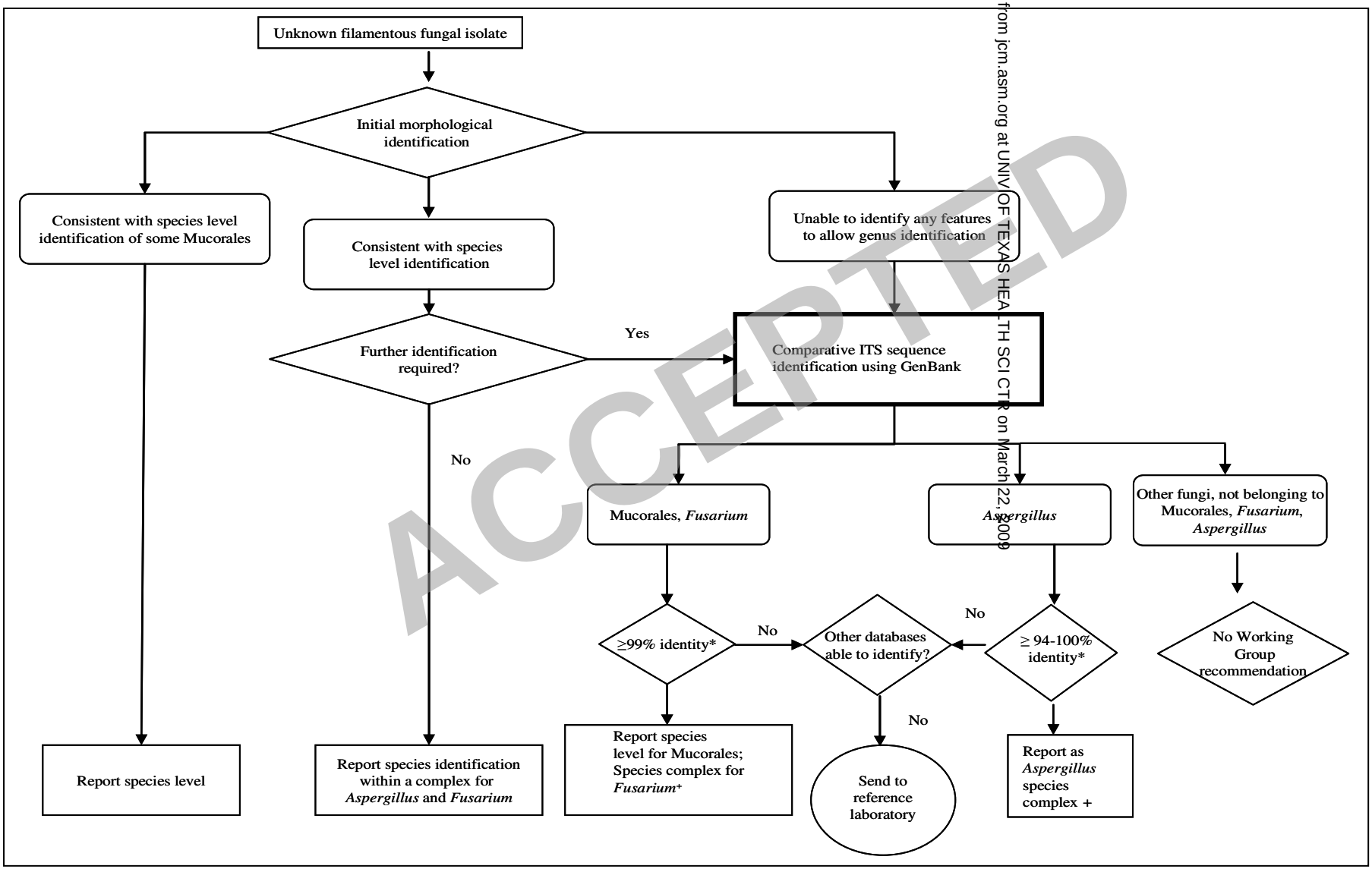
582

Figure 2



*Many factors affect percent identity scores including quality and length of query sequence, the number and accuracy of existing GenBank records for same species and locus.

+ Identification to species within the *Aspergillus* and *Fusarium* complex can be achieved by comparative sequence analyses of protein coding regions.

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Interlaboratory reproducibility of a single locus sequence-based method for strain typing of *Aspergillus fumigatus*

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Abstract

Seven international laboratories tested the recently proposed single locus typing strategy for *A. fumigatus* sub-typing for interlaboratory reproducibility. Comparative sequence analyses of portions of the locus AFUA_3G08990, a putative cell surface protein (denoted as CSP), was performed with a panel of *Aspergillus* isolates. Each laboratory followed very different protocols for extracting DNA, PCR, and sequencing. Results revealed that the CSP typing method was a reproducible and portable strain typing method.

Strain typing of *Aspergillus fumigatus* can be important for detecting outbreaks and in epidemiological investigations. Recently, a novel, simple and rapid single locus sequence typing strategy has been proposed as a typing tool for *A. fumigatus* (2). Genetic diversity in this locus arises from both tandem repeats and point mutations of the gene encoding the putative Cell Surface Protein (CSP), AFUA_3G08990 (2). Balajee et al employed this method (denoted as CSP typing) to sub-type 55 epidemiologically linked *A. fumigatus* isolates obtained from six nosocomial outbreaks of invasive aspergillosis (IA) and found the technique satisfied the tenets of a good sub-typing method as it identified distinct genotypes as well as clusters of closely related isolates (clonal complex). Although a subsequent study found that CSP typing had a lower discriminatory power when compared to a microsatellite based method, CSP typing remains useful as a quick frontline strategy for *A. fumigatus* strain discrimination (1). Importantly, since CSP typing employs a comparative sequencing strategy, it does not require elaborate training or software for analyses, is relatively user-friendly, economical and therefore amenable for use in reference microbiology laboratories. Other available sub-typing methods such as microsatellite (e.g. Str*Af*) based assays (3), *Afut1* DNA hybridization profiles (*Afut*1 method) (4) have superior discriminatory power but need specialized equipment and dedicated software. And, since reproducibility studies have not been conducted using these techniques, the data obtained cannot be readily shared between laboratories.

Balajee et al evaluated the CSP typing method for typeability, in vitro stability, intralaboratory reproducibility, and concordance with other typing methods (2). However,

the interlaboratory reproducibility of this method has not been tested so far. Given that one of the hallmarks of a good typing method is reproducibility which is independent of the operator, place and time (5), we examined the reproducibility of CSP typing in diverse laboratory settings with data generated under a wide array of experimental conditions.

To test interlaboratory comparability, a panel of *A. fumigatus* isolates were selected from outbreak isolates whose CSP genotypes were established in a previous study (1, 2). In brief, *A. fumigatus* isolates used in this study were obtained from previous cases of in invasive aspergillosis outbreaks and represented both clonal and distinct genotypes (as verified by CSP typing, *Afut1* and STR*Af* methods (1, 2)). Species identification of all *A. fumigatus* isolates was confirmed by sequence comparison of the β tubulin region {Balajee, 2007 #372}.

The panel consisted of fourteen *A. fumigatus* isolates: five isolates shared the same CSP type (arbitrarily designated genotype 1), eight isolates shared another CSP type (genotype 2), while one isolate had a unique CSP type (genotype 3). In addition, one isolate of *A. flavus* (CDC 14) was included as an outlier. Isolates were randomly coded, sub-cultured on Sabouraud dextrose agar slants, and then sent to seven international laboratories which represented research, clinical, and reference facilities. Each laboratory was also provided with the following *A. fumigatus* specific primers: 5'-TTGGGTGGCATTGTGCCAA (forward), 5'- GGAGGAACAGTGCTGTTGGTGA (reverse). These primers amplify a ~550 to ~700 bp fragment of the AFUA_3G08990

gene (dependent on the number of repeats). The participating laboratories cultured, isolated DNA, and performed PCR, sequencing, and DNA sequence analysis using their own routine methods.

The participating laboratories were requested to (i) generate CSP sequences from the panel of isolates and align the sequences using the Af293 (*A. fumigatus* isolate whose genome has been completely sequenced) CSP sequence as a reference (GenBank accession XM_749624), (ii) visually identify unique and shared genotypes, (iii) assign arbitrary designations to each distinct CSP genotype represented by one or more isolates in the panel – for example if isolates 1, 2, and 3 were observed to have related genotypes, they were assigned to genotype X, (iv) submit the arbitrary genotype assignments and all sequences in FASTA format via email to the coordinating laboratory, and (v) send detailed protocols on the methods used to generate the sequences to the coordinating laboratory. Each participating laboratory cultured, isolated DNA, performed PCR, sequencing, and DNA sequence analysis using methods which were routine in their individual laboratories.

Culture methods included seven different media (both broth and agar based) and two incubation temperatures (30ºC and 37ºC). For DNA extraction, two laboratories harvested mycelial mats and five harvested mycelia and spores from plates. One laboratory only collected spores for DNA isolation. DNA isolation utilized a variety of methods including commercially available kits as well as in-house protocols. Only one laboratory quantitated the isolated genomic DNA and made working dilutions of equal

concentration (10 ng/µl), while the others used the genomic DNA directly in the PCR reaction, regardless of concentration.

PCR cycling was performed on four models of thermal cyclers from three manufacturers. PCR amplifications were accomplished with either commercially available kits (one laboratory), or in-house PCR mixes (six laboratories), utilizing four different polymerases. All laboratories visualized the PCR products on an agarose gel, either commercial or made in-house. PCR product clean-up was performed either by the ExoSap enzyme reaction (one laboratory), magnetic beads (one laboratory) or by column purification (five laboratories). Five laboratories estimated the concentration of purified PCR products by comparison to a commercial mass ladder standard on an agarose gel, while two laboratories utilized the NanoDrop UV reader (Thermo Scientific) to quantitate the PCR products. One laboratory used the PCR products regardless of the concentration. Sequencing was performed using either Applied Biosystems BD 3.1 or BD 1.1 Dye Terminator chemistry or DYEnamic ET Dye terminator chemistry (GE Healthcare) on three different models of capillary electrophoretic sequencers. One laboratory utilized a commercial sequencing service. All laboratories sequenced both the forward and reverse strands and sequence editing was performed using Sequence Analyzer, Contig Express, MacVector, Sequencher, or BioEdit software packages. Sequence alignments were assembled using the BioEdit 7.0.9, ClustalX 1.83, Lasergene 8.0 or Mega 4.0 software.

Despite the wide spectrum of reagents, equipment and methods used to obtain the CSP sequences, five laboratories assigned the correct genotype to all isolates, thus yielding

100% concordance (Table 1), while laboratory 5 and 6 reported a concordance of only 93% and 85% respectively. Laboratory 5 reported the sequence from isolate CDC 3 as genotype 1, when the correct designation for this isolate was genotype 2. Similarly, laboratory 6 identified the isolate CDC6 as genotype 2, when the correct designation should have been genotype 3. The sequences obtained by these laboratories were of high quality and identical to those of the genotypes that were incorrectly assigned. All other sequences generated by these laboratories were also of high quality with no base-call errors. Therefore, we speculate that laboratories 5 and 6 may have reported incorrect genotype designations because of possible cross-contamination with another isolate from the *Aspergillus* panel. Alternatively, this could be also be attributed to an inadvertent exchange of samples that may have occurred at any stage of the process from culturing of the organism, DNA extraction, to PCR or sequencing. Six laboratories reported that isolate CDC14 yielded no PCR product; this was expected since this isolate was *A. flavus*, and should not be amplifiable with the primer set provided. Laboratory 6 reported this isolate's genotype as belonging to genotype 1, reiterating the likelihood of contamination problems in this laboratory.

The participating laboratories aligned the sequences and assigned genotype scores by visual inspection as described previously (2). The number of isolates in the panel was relatively small, and the differences in repeat number are easy to see in aligned sequences. However, this type of visual analysis would be difficult in larger studies and a more robust, objective genotype scoring system which would remove any potential for human error in genotype assignment should be developed for such analyses.

Interestingly, the limiting factor of this typing strategy was strain contamination and/or human error involving sample exchange, rather than sequencing errors or subjective data interpretation. *Aspergillus* spores are easily aerosolized and extreme care must be taken when working with these organisms to prevent contamination. Assuming that appropriate precautions are taken to prevent contamination, we demonstrate here that CSP typing performed in different laboratories was concordant and results can therefore be compared directly, despite considerable variation in protocols.

Recently, the STR*Af* method was demonstrated to have good interlaboratory reproducibility for *A. fumigatus* sub-typing (4). In this study where five laboratories participated, non-specific amplification products, bleed-through of the different fluorescent labels and inexperience of laboratories lead to some inconsistencies in results. Here, we present results of another multicenter study for *A. fumigatus* sub-typing that also had superior reproducibility. Such multi-laboratory reproducibility studies are essential to ensure that any proposed sub-typing method can be reliably employed for epidemiological studies.

Additionally and importantly, all data in this study were shared via the internet, thus confirming that the CSP typing scheme can be a portable and thereby a convenient strategy for interlaboratory data sharing or comparison. Furthermore, the data from such studies can easily be stored in a database and archived, retrieved, and reanalyzed at any time, making this a useful tool for global molecular epidemiological investigations of *A.*

163 *fumigatus*. The use of inexpensive or free web-based software for data analysis makes
164 this an attractive tool for small or cost-conscious laboratories. In summary, this
165 international, multi-laboratory study confirms the reproducibility and portability of the
166 CSP typing method.

Disclaimer

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the CDC.

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Table 1. CSP genotypes assigned to the *Aspergillus* panel, as reported by participating laboratories.

Isolate	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
CDC 1	2	2	2	2	2	2	2
CDC 3	2	2	2	2	1*	2	2
CDC 4	1	1	1	1	1	1	1
CDC 5	2	2	2	2	2	2	2
CDC 6	3	3	3	3	3	2*	3
CDC 7	2	2	2	2	2	2	2
CDC 8	1	1	1	1	1	1	1
CDC 9	1	1	1	1	1	1	1
CDC 10	1	1	1	1	1	1	1
CDC 12	2	2	2	2	2	2	2
CDC 14	NP	NP	NP	NP	NP	1*	NP
CDC 15	2	2	2	2	2	2	2
CDC 19	2	2	2	2	2	2	2
CDC 20	2	2	2	2	2	2	2
CDC 21	1	1	1	1	1	1	1

Each of the laboratories assigned a genotype number to all *A. fumigatus* isolates (except CDC 14 which is *A. flavus*).
NP- no product; *Denotes incorrectly assigned genotype

References

1. Balajee, S. A., H. A. de Valk, B. A. Lasker, J. F. Meis, and C. H. Klaassen. 2008. Utility of a microsatellite assay for identifying clonally related outbreak isolates of *Aspergillus fumigatus*. J Microbiol Methods **73**:252-6.
2. Balajee, S. A., S. T. Tay, B. A. Lasker, S. F. Hurst, and A. P. Rooney. 2007. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell **6**:1392-9.
3. de Valk, H. A., J. F. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol **43**:4112-20.
4. de Valk H. A., J. F. G. M. Meis, S. Bretagne, J.-M. Costa, B. A. Lasker, S. A. Balajee, A. C. Pasqualotto, M. J. Anderson, L. Alcazar-Fuoli, E. Mellado and C. H. W. Klaassen. Interlaboratory reproducibility of a microsatellite-based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: proof of concept. Clin. Microbiol. Infect. 2009, In Press
5. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J. Clin. Microbiol. **40**:2886-92.
6. van Belkum, A., P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, S. Brisse, and M. Struelens. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. **13** Suppl. 3:1-46.

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Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola,* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*)

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Abstract

Isolation and characterization of the new species *Chrysosporium ophiodiicola* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*) is reported. The analysis of the sequences of different fragments of the ribosomal genes demonstrated that this species belongs to the Onygenales and that it is genetically different from other morphologically similar species of *Chrysosporium*. This new species is unique in having both narrow, cylindrical to slightly clavate conidia, and a strong pungent odor.

Keywords: snake, granuloma, *Chrysosporium ophiodiicola*

CASE REPORT

A black, male rat snake (*Elaphe obsoleta obsoleta*) of undetermined age was presented with a history of prolonged anorexia and slow growing facial masses. The snake was found as an adult at an old home site in an old barn near Sparta, Georgia by the current owner, a wildlife educator. The snake had been in his possession for four years and was frequently used in public educational performances in the southeast. Upon presentation, the snake had a 1cm x 1.5 cm subcutaneous longitudinally ovoid swelling overlying his right ventral mandible area (Fig. 1A). He also had a 1cm swelling overlying his right eye and extending down into the orbit, displacing the eyeball laterally, and displacing the palate and dorsal limit of the choana ventrally. The masses were lobular, whitish in appearance and enclosed in a thin capsule. The submandibular mass was removed in its entirety, as its capsule was very discrete. The other mass was very friable and locally extensive. Both masses were surgically removed and submitted for histopathological examination and culture. However, all portions of the second mass could not be completely removed due to its location, but the area enclosing it was debrided. At the time of surgery, the snake was treated with meloxicam ((Metacam, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO)) at a dose of 0.2mg/kg SID, enrofloxacin (Baytril, Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, Kansas) at a dose of 5mg/kg BID. This was continued until the histopathology report indicating a fungal infection was received. The enrofloxacin was discontinued and ketoconazole was initiated. Single oral administration of ketoconazole (Apotex, Inc., Toronto, Ontario) 50mg/kg was administered daily. The snake was kept at 29.5^{o}C and was tube fed Hill's Prescription Diet (Hill's Pet Nutrition, Inc., Topeka, KS) A/D 25ml every 3 days. There was a moderate amount of postoperative swelling at the incision

over the orbit. This was treated with warm wet compresses daily and the swelling decreased. The snake passed away 2 months after surgery.

The histopathological evaluation and primary culture was performed at the University of Georgia, Veterinary Diagnostic and Investigational Laboratory, Tifton, GA, USA under the accession 38816-06. Both masses consisted of multifocal to coalescing granulomas. The granulomas had central regions of amorphous eosinophilic and occasional cellular debris surrounded by an inflammatory cell infiltrate consisting of histiocytes, lymphocytes and occasional heterophils (Fig. 1B). Mild concentric fibrosis was surrounding these areas. Moderate numbers of hyphae and closely segmented arthroconidiating hyphae were found primarily within the centers of the granulomas. The hyphae were 3-7 µm broad, parallel-walled, segmented, and occasionally branching. Similar fungal structures were also observed with use of a Grocott-Gomori's methenamine silver stain (Fig. 1C).

Routine bacterial and fungal cultures were performed from the tissue sample. For fungal culture, a portion of the sample was inoculated on a Sabouraud dextrose agar (Remel, Lenexa, KS) and incubated at 29^{o}C for four weeks. Bacterial cultures were negative. A moderate to heavy and pure growth of a fungus was observed on fungal medium. Colonies were white in color and had sterile septate hyphae and no fruiting bodies were present. The fungus was unidentifiable using conventional laboratory techniques. The isolate was forwarded to the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA, for morphologic identification and accessioned into the culture collection as UTHSC 07-604.

On potato flakes agar (PFA – prepared in-house) (12) at 23ºC colonies were white to pale yellow with a similar reverse, velvety to granular with age, microscopically resembled a *Chrysosporium*, displayed conidia borne on stalks as well as arthroconidia, and produced a strong pungent odor. As the isolate did not appear to morphologically match any known *Chrysosporium* species, it was submitted to Department of Microbiology and Immunology for molecular characterization under accession number R-3923.

The ITS and D1/D2 regions were amplified using the DNA preparation methodology, primers, and PCR conditions as previously described (5, 12). PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced at the Advanced Nucleic Core Facility of the University of Texas. Each sequence was then used to search GenBank using the BLASTn algorithm at http://www.ncbi.nlm.nih.gov/. Sequencing of the ITS (614 bp length, accession # EU715819) and D1/D2 (486 bp length, accession # EU715820) regions failed to provide an unequivocal identification as the closest D1/D2 maximum identity was 93% (*Onygena corvina*, accession # AB075355) and the closest ITS maximum identity was 84% (*Arthroderma multifidum*, accession # AB361651). However, sequence data confirmed the association of the clinical isolate with the onygenalean fungi. As the percentages of similarity with all the sequences deposited in the GenBank were very low, a conclusive identity could not be made. The isolate was forwarded to the Mycology Unit at Rovira i Virgili University in Reus, Spain, where further extensive morphologic and molecular phylogenetic studies were undertaken to characterize this fungus.

The morphological description of the present isolate is as follows:

Colonies on potato carrot agar (PCA; potato 20 g, carrot 20 g, agar 15 g, water 1L) (Fig. 2C) attained 27-29 mm diameter in 14 days at 25ºC, and were white with uncolored reverse. They were felty, plane and fimbriate with a poorly defined margin. Sparse tufts of aerial mycelium were present on the submarginal zone. Vegetative hyphae were hyaline, branched, septate, smooth and thin-walled. They were 1.5-2.5 µm wide, and often disarticulating at maturity to form cylindrical, 7.5-10 x 2 -2.5 (3) µm arthroconidia adjacent to each other. Fertile hyphae arise as lateral branches. Terminal and lateral conidia were borne on straight or flexuose side branches of variable length (4.5-16 µm) or were sometimes sessile. Conidia were unicellular, solitary, thin-walled, smooth, hyaline to pale yellow, cylindrical to slightly clavate, (4.0-6.5 (9) x 2.0-3.0 µm) and were released by rhexolytic dehiscence, with broad and long basal scar (Figs. 2D-F) . Intercalary solitary conidia were often present, similar to the terminal and lateral ones. Racquet hyphae were scarce and chlamydospores were not observed. On potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) the fungus grew more quickly and produced more dense colonies, 31- 35 mm diameter in 14 days at 25ºC (Fig. 2B). They were white to pale yellow, buff after one month, powdery, with droplets of colorless or light yellow exudates at the periphery. On phytone-yeast extract agar (PYE, BBL, Cockeysville, USA) the colonies had 32-39 mm diameter in 14 days at 25º C (Fig. 2A), and they were white and light yellow at the centre, powdery and dense, with the presence of droplets of colorless exudate at the centre and a light brown reverse. On oat-meal agar (OMA; 30 g oat flakes, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 15 g agar, 1 L tap water), the colonies were similar to those on PCA. The fungus had a very restricted growth at 15ºC (5 mm diameter in14 days). At 37ºC there was no growth. The colonies produced a strong pungent (skunk like) odor after one month of incubation in all the media tested. Attempts to induce the teleomorph on OMA and sterile garden soil to which horse hair had been

added were unsuccessful after two months of incubation at 25ºC. However, a strong keratinolytic activity was noticed.

The main characteristics of the snake isolate were the presence of numerous narrow cylindrical to slightly clavate conidia and the strong pungent odor of the colonies. This odor is not rare in the Onygenales since other species such as *Chrysosporium mephiticum* Sigler and *Aphanoascus mephitalis* (Malloch & Cain) Cano & Guarro show similar characteristics (12). However, these species can be easily differentiated from the present fungus by their morphology; *C. mephiticum* has pyriform to subglobose conidia occurring more or less synchronously and *A. mephitalis* usually produces the teleomorph in culture and has a *Malbranchea* anamorph. In addition, these species show very different ITS sequences (4, 6, 14) (Fig.3). Narrow cylindrical conidia are also produced by *Chrysosporium europae* Sigler, Guarro & Punsola. But this species can be easily differentiated from the new species by its characteristic vinaceous buff pigmented colonies on PYE and the absence of strong pungent odour (11).

The combined morphological, cultural and molecular characteristics of the snake isolate do not correspond to any of the species described to date within the genus *Chrysosporium*. Thus, the new following new species is proposed:

Chrysosporium ophiodiicola Guarro, D.A. Sutton, Wickes, and Rajeev, **sp. nov.**

Etym.: from the Greek *ophio,* snake.

Ad fungos conidiales, hyphomycetes pertinens.

Coloniae in agaro cum decocto tuberorum et carotarum (PCA) post 14 dies ad 25 C, 27 – 29 mm diametro celeriter crescentes, planae, albae; reversum hyalinae. Coloniae in agaro cum decocto tuberorum (PDA) post 14 dies ad 25 C, 31 – 35 mm diametro; in agaro phytone extracto levedinis (PYE)

post 14 dies at 25 C, 32-39 mm diametro. Ad 37 C incrementum nullum. Odor foetidus. Hyphae hyalinae vel subhyalinae, leviter ramosae, septatae, 1.5 – 2.5 µm latae. Conidia terminalia et lateralia sessilia vel in ramae laterales, cylindrica vel clavatae, hyalina vel lutea, leviatunicata, 4.0 – 6.5 (1) x 2.0 – 3.0 µm; arthroconidia hyalina vel lutea, leviatunicata, cylindrica, 7.5 – 10 x 2 – 2.5 (3) µm. Chlamydosporae absunt. Teleomorphosis ignota. Species keratinolytica *Cultura typica: ex ophio pelle .In collectione fungorum CBS* 122913 *deposita est. Isotypus* FMR 9510, UTHSC 07-604.

The phylogenetic analysis of the ITS region of *C. ophiodiicola* and other related onygenalean fungi was performed with MEGA 2.1 software (7), using the Neighbor-joining (NJ) method and based on Kimura's 2-parameter corrected nucleotide distances. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* was the nearest species to *C. ophiodiicola* in the ITS neighbor-joining tree (Fig. 3). Both species are associated with infections in reptiles.

Chrysosporium ophiodiicola was isolated from a subcutaneous granuloma of a snake, which is not an unusual source for recovering chrysosporia. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* has been isolated from cases of dermatitis in snakes (2, 15) chameleons (9), crocodiles (13), bearded dragons (3) and from a nasal granuloma in an Ameiva lizard (8). In a recent report a *Chrysosporium* species related to *Nannizziopsis vriesii* has been isolated from a case of cutaneous hyalohyphomycosis from two green iguanas (1). Phenotypically *C. ophiodiicola* can be separated from *Chrysosporium* anamorph of *Nannizziopsis vriesii* by the absence of asperulate fertile hyphae, globose to pyriform conidia sometimes grouped in clusters, and the presence of odor in the colonies of the former.

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REFERENCES

1. **Abarca, M.L., J. Martorell, G. Castella, A. Ramis, and F.J. Cabanes.** 2008**.** Cutaneous hyalohyphomycosis caused by a *Chrysosporium* species related to *Nannizziopsis vriesii* in two green iguanas (*Iguana iguana*). Med. Mycol. **46:** 349-354.

2. **Bertelsen, M.F., G.J. Crawshaw, L. Sigler, and D.A. Smith.** 2005. Fatal cutaneous mycosis in tenacled snakes (*Erpeton tentaculatum*) caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii.* J. Zoo. Wildl. Med. **36:**82-87.

3. **Bowman, M.R., J.A. Paré, L. Sigler, J.P. Naeser, K.K. Sladky, C.S. Hanley, P. Helmer, L.A. Phillips, A. Brower, and R. Porter.** 2007. Deep fungal dermatitis in three inland bearded dragons (*Pogona vitticeps*) caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii.* Med. Mycol. **45:**371-376.

4. **Cano J., M. Sagués, E. Barrio, P. Vidal, R.F. Castañeda, J. Gené, and J. Guarro.** 2002. Molecular taxonomy of *Aphanoascus* and description of two new species from soil. Stud. Mycol. **47:**153-164.

5. **Drees, M., B.L.Wickes, M. Gupta, S. Hadley.** 2007. *Lecythophora mutabilis* prosthetic valve endocarditis in a diabetic patient. Med. Mycol. **45:**463-467.

6. **Garg, A.K.** 1966. An addition to the genus *Chrysosporium* Corda. Mycopathol. Mycol. Appl. **30:**221-224.

7. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics **17:**1244-1245.

8. **Martell, A., P.A. Fonteyne, K. Chiers, A. Decostere, and F. Pasmans.** 2006. Nasal *Nannizziopsis vriesii* granuloma in an Ameiva lizard (*Ameiva chaitzami*). Vlaams Diergeneeskundig Tijdschrift 75: 30-307.

9. **Paré, J.A., K.A. Coyle, L. Sigler, A.K. Maas, and R.L. Mitchell.** 2006. Pathogenicity of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* for veiled chameleons (*Chamaeleo calyptratus*). Med. Mycol. **44:**25-31.

10. **Rinaldi, M.G.** 1982. Use of potato flakes agar in clinical mycology. J. Clin. Microbiol. **15:**1159-1160.

11. **Sigler, L., J. Guarro, and L. Punsola.** 1986. New keratinophilic species of *Chrysosporium.* Can. J. Bot. **64:**1212-1215.

12. **Sutton, D.A., B. L.Wickes, E.H.Thompson, M.G. Rinaldi, R.M Roland, M.C. Libal, K. Russel, S. Gordon.** 2008. Pulmonary *Phialemonium curvatum* phaeohyphomycosis in a Standard Poodle dog. Med. Mycol. **46:**355-359.

13. Thomas, A.D., L. Sigler, S. Peucker, J.H. Norton, and A. Neilan. 2002. *Chrysosporium* anamorph of *Nannizziopsis vriesii* associated with fatal cutaneous mycoses in the salt water crocodile (*Crocodylus porosus*). Med. Mycol. **40:**143-151.

14. Vidal, P., and J Guarro. 2002. Identification and phylogeny of *Chrysosporium* species using RFLP of the rDNA PCR-ITS region. Stud. Mycol. **47:**189-198.

15. Vissiennon, T., K. F. Schuppel, E. Ullrich, and A.F.Kuijpers. 1999. Case report. A disseminated infection due to *Chrysosporium queenslandicum* in a garter snake (*Thamnophis*). Mycoses. **42:**107-110.

FIGURE LEGENDS

Fig. 1

1A: Cutaneous masses

1B: H&E stained section of the lesion

1C: GMS stained section of the lesion

Fig. 2

Colonial and microscopic morphology of ***Chrysosporium ophiodiicola*** R-3923**.**

2A: Phytone yeast-agar, front and reverse;

2B:Potato dextrose agar, front and reverse;

2C:Potato carrot agar, front and reverse;

2D:Fertile hyphae and conidia;

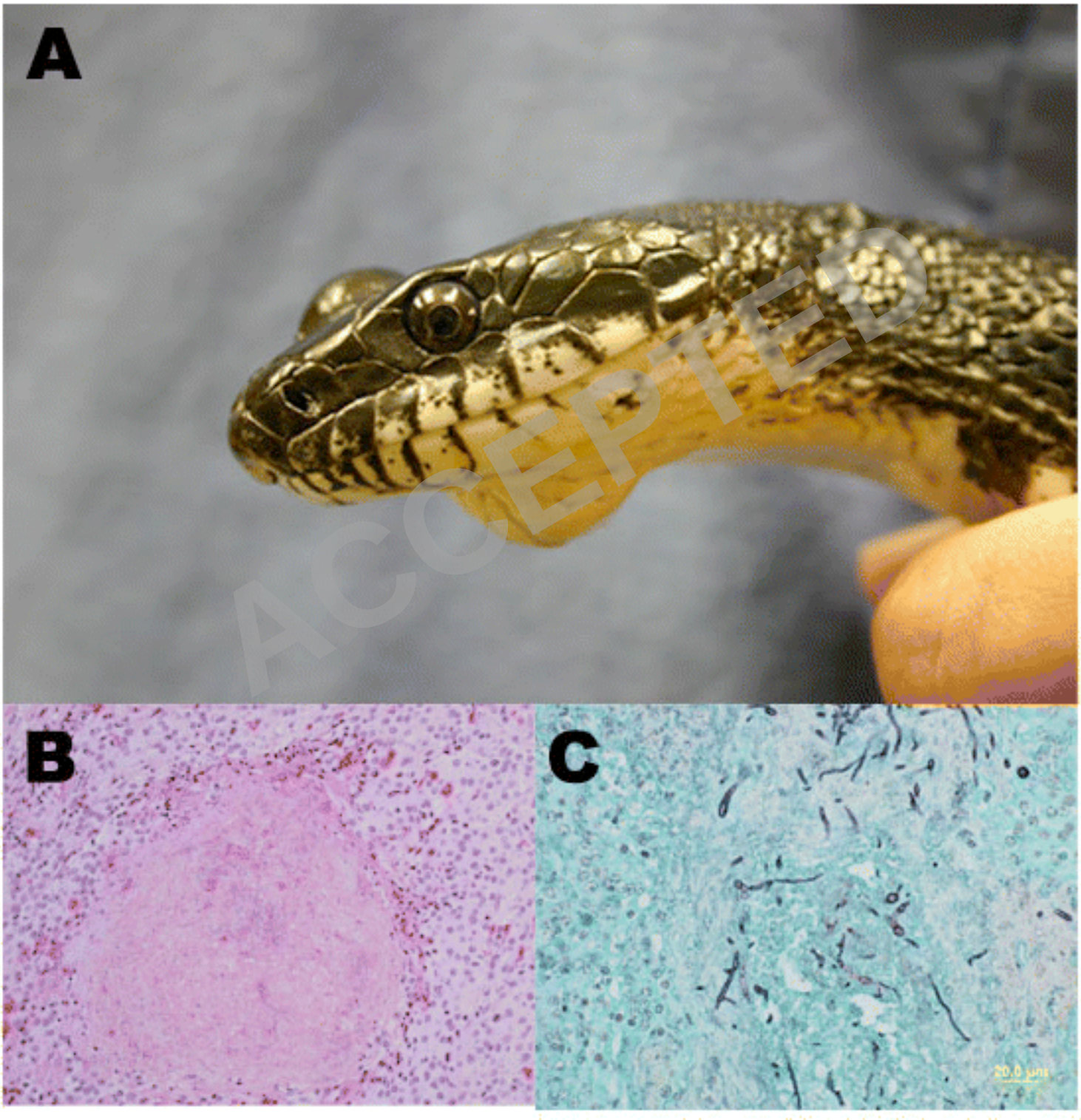
2E: Conidia showing remnants of wall following rhexolytic dehiscence;

2F: Fertile hyphae with arthroconidia and, terminal and lateral conidia.

Fig. 3.

Neighbor-joining tree based on Kimura 2-p corrected nucleotide distances among ITS1-5.8s ITS2 rDNA sequences of the species compared with *Chrysosporium ophiodiicola*. Branch

lengths are proportional to distance. Bootstrap replication frequencies over 70% (1000 replications) are indicated on the nodes. Abbreviations: *A.*, *Aphanoascus*; *C.*, *Chrysosporium*; *Cor.*, *Corynascus*; *N.*, *Nannizziopsis*; an., anamorph.


A
B
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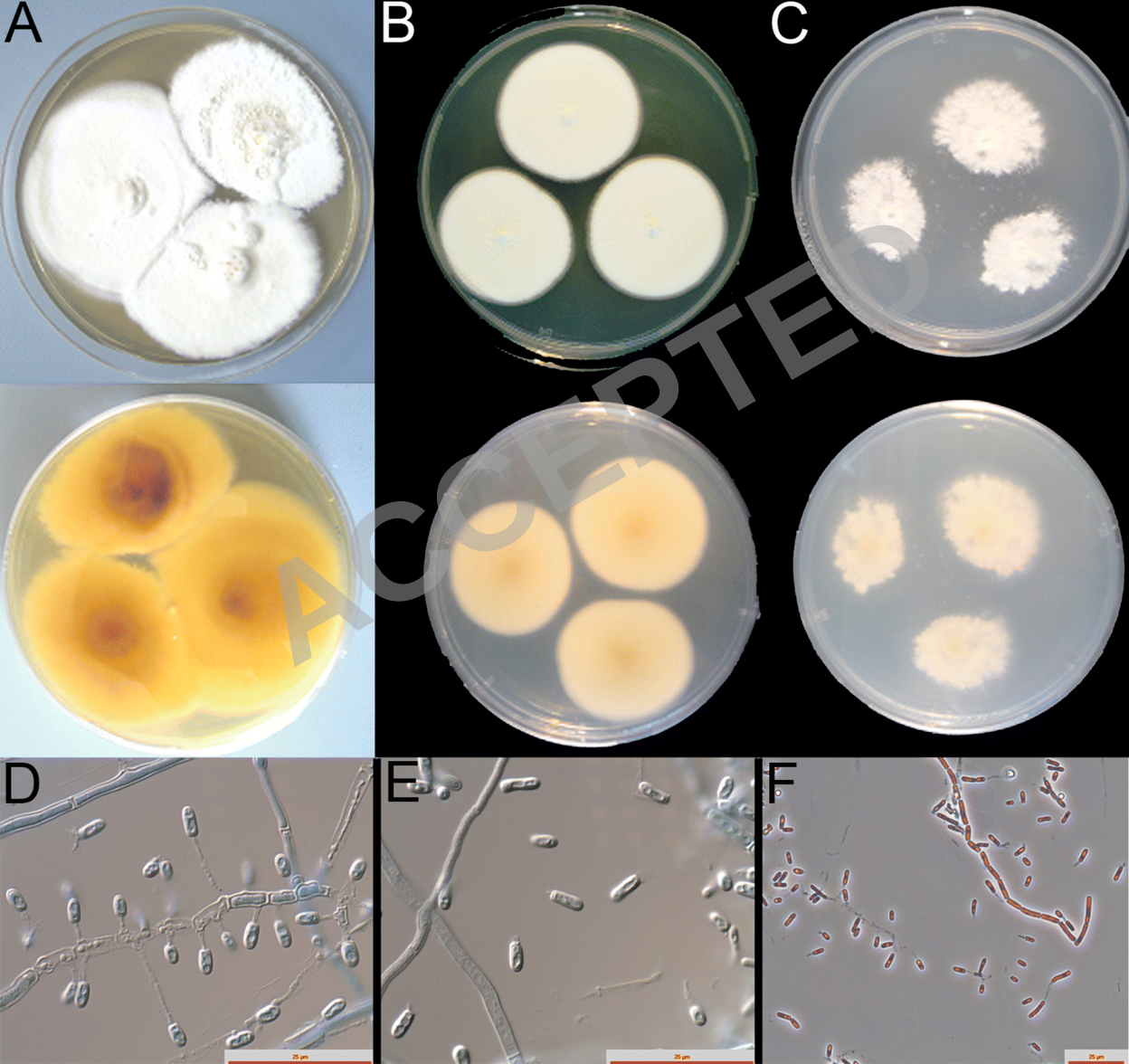

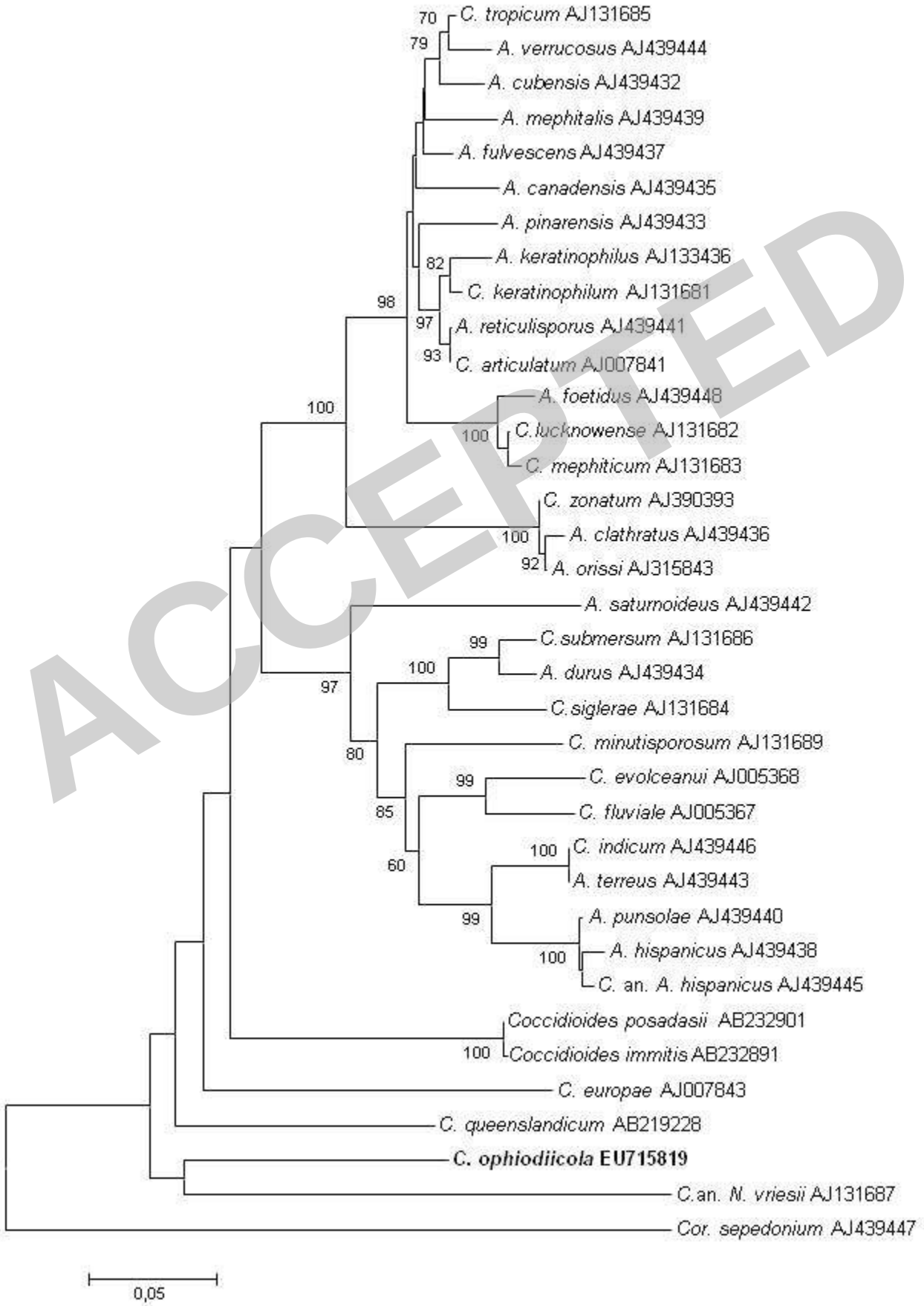
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79
C. tropicum AJ131685
A. verrucosus AJ439444
A. cubensis AJ439432
A. mephitalis AJ439439
A. fulvescens AJ439437
A. canadensis AJ439435
A. pinarensis AJ439433
82
A. keratinophilus AJ133436
C. keratinophilum AJ131681
98
97
A. reticulisporus AJ439441
93
C. articulatum AJ007841
100
A. foetidus AJ439448
100
C. lucknowense AJ131682
C. mephiticum AJ131683
C. zonatum AJ390393
100
A. clathratus AJ439436
92
A. orissi AJ315843
A. saturnoideus AJ439442
99
C. submersum AJ131686
100
A. durus AJ439434
97
C. siglerae AJ131684
80
C. minutisporosum AJ131689
99
C. evolceanui AJ005368
85
C. fluviale AJ005367
100
C. indicum AJ439446
60
A. terreus AJ439443
99
A. punsolae AJ439440
100
A. hispanicus AJ439438
C. an. A. hispanicus AJ439445
Coccidioides posadasii AB232901
100
Coccidioides immitis AB232891
C. europae AJ007843
C. queenslandicum AB219228
C. ophiodiicola EU715819
C. an. N. vriesii AJ131687
Cor. sepedonium AJ439447
0,05

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Strain-Dependent Variation of 18S rDNA Copy Number in *Aspergillus fumigatus*

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Keywords: Taqman, quantitative real time PCR, aspergillosis, ribosomal

Running Title: *Aspergillus fumigatus* rDNA copy number

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ABSTRACT

Enumerating *Aspergillus fumigatus* colony forming units (CFU) can be challenging since CFU determination by plate count can be difficult. CFU determination by quantitative real time PCR (qPCR), however, is becoming increasingly common and usually relies on detecting one of the subunits of the multicopy ribosomal RNA genes. This study was undertaken to determine if rDNA copy number was constant or variable among different *A. fumigatus* isolates. *FKS1* was used as a single copy control gene, and was validated against single copy (*pyrG* and *ARG4*) and multi copy (*arsC*) controls. Copy number of the 18S rDNA subunit was then determined for a variety of isolates and was found to vary with strain, from 38-91 copies per genome. Investigation of the stability of 18S rDNA copy number after exposure to a number of different environmental and growth conditions, revealed that the copy number was stable, varying less than one copy across all conditions including isolates recovered from an animal model. These results suggest that while the ribosomal genes are excellent targets for enumeration by qPCR, the copy number should be determined prior to using them as targets for quantitative analysis.

INTRODUCTION

Aspergillosis is caused by pathogenic fungi in the genus *Aspergillus* and includes allergic, superficial, saprophytic and invasive disease (12). The frequency of invasive aspergillosis (IA) continues to increase due to a growing population of immunosuppressed individuals. In fact, *A. fumigatus*, the most frequent *Aspergillus* species in IA cases (19), is now the most common airborne human fungal pathogen (25). Once infected, the mortality rate from IA can be unacceptably high for some patient populations, ranging from 70-90% depending on the patient type (7, 13, 31). However, in spite of the severity of disease, the ubiquitous nature of *Aspergillus* in the environment makes exposure difficult to avoid, consequently susceptible patients will almost always be at risk for infection.

The life-threatening nature of IA makes accurate diagnosis and early detection crucial. Quantitative real time PCR (qPCR) is emerging as a sensitive and cost-efficient technique for detecting *Aspergillus* spp. from a diverse variety of sources, including clinical specimens. Investigators studying IA with animal models routinely use qPCR to measure fungal load (17, 27), including response to drug treatment (6, 42). Bioaerosol quantitation of *Aspergillus* spp., particularly in the hospital environment, is also amenable to qPCR (32). Finally, even though qPCR is not the first choice for clinical diagnosis of IA, it has proven useful for quantitating *Aspergillus* spp. from a variety of patient specimens (2, 26, 37) and has proven extremely useful as a secondary assay for comparative purposes during assay development (8, 23).

One of the drawbacks of PCR-based detection methods is a lack of standardization (5) and one of the first areas to standardize is selection of an appropriate target for amplification. The quantitative nature of qPCR allows an estimation of colony forming units (CFU) by equating the copy number of the target sequence with genome number through a simple ratio, provided the ratio remains invariant. In fungi the ribosomal genes have proven to be useful PCR targets

because of their sequence conservation, which has allowed the use of universal primers that enable the amplification of targets from unknown species. A second advantage of using the rDNA genes as an amplification target is the copy number, which can be 10-100x that of single copy genes (29, 30). However, in *A. fumigatus*, it is unclear whether all strains have the same number of rDNA subunits units. In other fungi, rDNA copy number is known to vary (4, 15, 16, 20, 29), although these observations have been made in fungi that are not frequently recovered as human pathogens. Given what is known in other organisms about the variability of rDNA copy number and the importance of *A. fumigatus* as a human pathogen, this study was performed in order to determine if rDNA copy number is constant or strain-specific in *A. fumigatus.*

MATERIALS AND METHODS

Strains and Media Strains used in this study are shown in Table 1 and were confirmed to be *A. fumigatus* by colony morphology and DNA sequencing of the ITS and D1/D2 regions. Each strain was grown on Sabouraud's dextrose (SAB) (Difco Laboratories, Detroit, MI) or Potato Dextrose (PD) broth or agar (Fisher Scientific, Pittsburgh, PA) for all assays, unless otherwise indicated. Agar media was prepared from broth by solidification with 2% agar. RPMI 1640 without L-glutamine (Mediatech, Inc., Herndon, Va) was prepared by filter sterilizing and adding to an autoclaved solution of 2% dextrose and 2% agar (BD Diagnostic Systems, Franklin Lakes, NJ).

DNA isolation Individual strains were inoculated into 200 ml of SAB broth in a 500 ml flask from a 7-day old suspension of ~5 x 10^8 conidia harvested from a PDA plate. The hyphae were recovered after 24 h by filtering through an 18.5 cm, 0.45-mm pore size Whatman disk

(Whatman, Florham Park, NJ), and washed with sterile saline. DNA isolation followed methods reported elsewhere (22, 41) with slight modifications. After the saline wash, approximately 200 mg of wet hyphae were briefly dried by blotting between Whatman paper (Whatman) and then placed into a sterile mortar and frozen for 10 min at -70°C. Fungal cell walls were mechanically broken by grinding with a pestle for 1-2 min after the addition of sterile sand and 2 ml of Masterpure Yeast DNA purification kit lysis buffer (Masterpure Yeast DNA purification kit, Epicentre Technologies, Madison, WI). The slurry was transferred to 2 x 1.5 ml microfuge tubes and spun at low speed (500 x g) for 15 sec to pellet the sand. Four hundred microliters of the supernatant were transferred to a 2.0 ml screw-capped microfuge tube and incubated at 65°C for 2 hours, after the addition of 6 µl of proteinase K (50µg/mL) from the DNA purification kit. Samples were processed from this point as described (22). After the final wash, the dried pellets were resuspended in 200 µl ultra pure water (Invitrogen, Carlsbad, CA). DNA was assessed for quality and quantified by gel electrophoresis and 260:280 nm absorbance ratio.

Due to the possibility of contamination of *Aspergillus* DNA with polysaccharides in crude DNA preps, DNA was further purified prior to performing qPCR assays. DNA was run in a 1.0% low melting-point agarose (InCert; FMC BioProducts, Rockland, Maine) gel to separate it from contaminating materials. Gel fragments containing DNA were recovered, placed into 1.5 ml microfuge tubes, and then treated with Gelase (Epicentre) according to manufacturer's instructions. Purified DNA was assessed and quantitated by spectrophotometer and agarose electrophoresis as above. Yields were 100 µg-500 µg.

Growth Conditions to Evaluate Stress Effect on rDNA Copy Number

In order to measure the effect of colony age on rDNA copy number, DNA was prepared from *A. fumigatus* strain AF293 grown for 3d, 5d, 10d, and 25d on PDA plates at 30°C. AF293 was also tested for the effect of temperature on copy number by preparing DNA from cultures grown at 30˚C and 45˚C for 5d on PDA plates. DNA was isolated and processed from each condition as previously described (22).

The effect of antifungal exposure on copy number was measured by harvesting AF293 grown in the presence of itraconazole (Oakdell Pharmacy, San Antonio, TX) using a modification of the standard MIC assay. Conidia were harvested from a 5 day old PDA plate grown at 30°C overnight and used to prepare inoculums containing 4.5 x 10^6 CFU/ml. Each inoculum (10 mL) was then grown overnight at 30°C in the presence of different itraconazole concentrations (0 µg/mL, 0.03 µg/mL, 0.06 µg/mL, .125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 2.0 µg/mL) under modified MIC conditions described by the National Committee for Clinical Laboratory Standards (38). DNA was then recovered as described above.

In order to determine what effect morphology had on copy number, AF293 DNA was isolated from pure conidia and hyphae. Conidial cultures were prepared from PDA plates grown for 11 days at 30°C and harvested by washing with 10 ml of sterile PBS-0.1% Tween 20. The suspension was pelleted by centrifugation at 4800 x g for 10 min. The supernatant was discarded and the conidial pellet was transferred to a 1.7 ml microcentrifuge tube and washed once with 500 µl of sterile water and once with 500 µl of 0.1 M $MgCl_2$. Hyphae were prepared as described (22). Conidial and hyphal DNA were recovered as described above.

The effect of growth *in vivo* during animal model infection on copy number was determined by passing AF293 through animals as follows. Non immunosuppressed mice and guinea pigs were infected as described by Sheppard *et al.* (34). Lungs and kidneys were

harvested 5d post infection. DNA was extracted from tissue according to the Standard Operating Procedures for Invasive Aspergillosis Animal Models (http://www.sacmm.org/sop.html) and recovered in 100 µl of QIAamp DNA mini-kit elution buffer (Qiagen, Valencia, Calif.). After quantitation, DNA was stored at -20°C until analyzed.

PCR and qPCR primer and probe design

The PCR primer and probe sequences used to quantitate and amplify *A. fumigatus* target genes are shown in Table 2. Primers for qPCR were designed using Primer Express Software v2.0, which is an application-based design software provided by ABI (Applied Biosystems, Inc., Foster City, CA), or were designed based on previously published reports. The primers and probe for the *A. fumigatus FKS1* gene were designed according to Costa *et. al.* (10). The primers and probe for the 18s rDNA sequence were also based on a previous study (6). The *FKS1* gene was chosen because it is a known single copy gene in *A. fumigatus* involved in β (1–3) glucan synthesis (3), and was used as an internal control. The *pyrG* gene, which encodes orotidine-5'-monophosphate decarboxylase, was also included as a second single copy reference gene (11, 44) and used to confirm *FKS1* copy number determination. *ARG4,* which encodes carbamoyl-phosphate synthase, was the third single copy reference gene used in this study and was identified from the genome sequence.

In order to test our ability to discriminate multiple copy genes, a duplicated gene was selected for analysis. The *arsC* (arsenate reductase) gene is a duplicated gene found in some but not all strains of *A. fumigatus*, and is present in the AF293 genome sequence as two copies (33). Since *arsC* is not present in all *fumigatus* strains, we reconfirmed that it was present in AF293 in two copies using a method independent of qPCR. Based on the sequences of *arsC* from the two

chromosomal locations, allele specific primers were designed that spanned an *Nco* I site within the coding sequence of each *arsC* allele (Table 2). The chromosome 1 *arsC* primers consisted of Ch1arsC.F and Ch1arsC.R. The chromosome 5 *arsC* primers consisted of Chr5arsC.F and Chr5arsC.R. Each allele was amplified using the following conditions, 94°C for 2 min, 32 cycles 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 2 min. The amplicons were then digested with 5U of *Nco* I (New England Biolabs, Beverly, MA) at 37°C for 3h and then separated on a 3% NuSieve GTG agarose gel (Cambrex Bio Science Inc., Rockland, ME). Sizes were then compared to the sizes predicted from the genome sequence.

qPCR Validation Assays and Calculations

FKS1, *pyrG*, *ARG4* (single copy genes), *arsC* (two copy gene) and 18s rDNA (multiple copies) gene copy number determinations were done by qPCR (Taqman) assay according to the method of Townson *et. al.* (39), with modifications. In order to determine the copy number of a variable gene (18s rDNA), a single copy reference gene needed to be identified and confirmed to be present in 1 copy/genome. Since the *FKS1* gene is highly conserved in fungi and has been shown in a number of reports to be present in single copy in *A. fumigatus* (3, 14, 33), we selected this gene to use as the single copy reference probe in the qRT-PCR reactions. Confirmation was performed by comparison to other *A. fumigatus* genes already known to be single copy. The single copy genes, *pyrG* and *ARG4*, were confirmed using relative quantification (ratios of one gene to another) to determine the number of copies present per genome. Quantification standards were run in conjunction with each set of samples after primers and probes for *FKS1*, 18S rDNA, *pyrG*, *ARG4*, *arsC* genes were optimized for template concentration and primer efficiencies (1).

qPCR reactions were performed in triplicate using an ABI/PRISM7900 Sequence Detector System (ABI) to detect MGB probe binding. *FKS1* was quantitated using both VIC and FAM dyes and used as a reference for comparison to the 18S rDNA FAM probe from each strain. Five serial 1:2 dilutions (20.0, 10.0, 5.0, 2.5, 1.25, 0.625ng/µl) of genomic DNA from *A. fumigatus* AF293 were used to generate standard curves of Ct (threshold cycle) value against the log DNA concentration on each PCR plate for the *FKS1* and 18S rDNA genes. Each experiment was performed three separate times from one DNA preparation and run in duplicate. Ct values were determined, and then converted into template quantity. After the creation of standard curves, copy numbers of each gene were determined by DNA quantification using Taqman technology. PCR cycle numbers were plotted against the value of 5' fluorescence signal, and then threshold values were plotted against the copy number of the template DNA, which were used to generate standard curves (1).

Absolute quantification using the ABI/PRISM7900 requires that the absolute quantities of the standards be determined by some independent means first. In this study fungal DNA was used to prepare absolute standards. Concentration and DNA quality were measured by A_{260} and gel electrophoresis, and converted to the number of copies using the molecular weight of the DNA. The equation Ct = m (log quantity) +b from the equation for a line (y = mx+b) was constructed by plotting the standard curve of log quantity versus its corresponding Ct value. If the curve demonstrated an r^2 value > 0.980 the standard curve then was used to determine sensitivity, primer efficiencies, and dynamic range, as well as specificity and reproducibility of every assay (*FKS1*, 18S rDNA, *pyrG*, *ARG4*, *arsC*). Amplification of serially diluted genomic DNA (standard curves) from *A. fumigatus* AF293 was repeated in triplicate, on different days, in order to test reproducibility, primer efficiencies, and DNA optimal dilutions for the rest of the

198 genes (*pyrG*, *ARG4*, and *arsC*). DNA concentrations ranged from 20.0 to 0.625ng/µl. Specificity
199 for all the assays was assessed by using DNA extracted from *Candida albicans* SC5314, as well
200 as mouse and guinea pig DNA (9, 28). Comparative copy numbers for confirmation experiments
201 were determined using the relative quantification (Delta Ct) $2^{-\Delta Ct}$ method. The 18S rDNA copy
202 numbers were determined by the absolute quantitation method, where total copies were first
203 calculated using: total 18S rDNA copies = 10((Ct-b)/m). The number of 18S rDNA copies per
204 genome were then determined by: 18S rDNA copies per genome = (total copies 18S
205 rDNA)/(total copies *FKS1*). Copy number was calculated as the ratio of template quantity for
206 18S rDNA to the template quantity for *FKS1*.
207
208 **Statistical Methods**
209 In each experiment, we altered one factor at a time under controlled conditions. This
210 approach minimized the sources of variability within an experiment and maximized statistical
211 power for detecting effects of a single factor on differential copy number. Results after
212 determination of 18s rDNA copy numbers were compared by the Wilcoxon rank sum test for
213 morphology and temperature. The Wilcoxon signed rank test was used to compare copy numbers
214 from different tissues in the same animal, and the Kruskal-Wallis test was used to compare
215 culture age and antifungal susceptibility. Statistical analysis was done at the University of Texas
216 Health Science Center at San Antonio Department of Epidemiology and Biostatistics. Two-tailed
217 *P* values less than 0.05 were considered significant.
218
219 **RESULTS**
220 *Copy number confirmation of FKS1*

A number of confirmatory assays were performed to verify that *FKS1* was present as a single copy in AF293. First, absolute quantitation was performed using *FKS1* probes labeled with two dyes; FAM and VIC. The slope of the VIC line was -3.9341 (from y=-39341x + 52.288) while the slope for the FAM line was -3.8971 (from y=-3.8971x + 51.593). The R^2 values of the VIC and FAM lines were .9946 and .9982 respectively, demonstrating that comparable results could be obtained independent of dye type. The copy number of *FKS1* was next determined in a subset of *A. fumigatus* strains (WSA-172, -445, -621, -419) by absolute quantitation using *FKS1* labeled with FAM and VIC for each strain. *FKS1* copy numbers determined by qPCR ranged from 0.93-1.10 copies and were rounded to 1 copy based on the close integer scoring method (18) so that it could be used as the single copy reference gene when determining copy number of other genes. We next compared the copy number of *FKS1* to other known single copy genes (*ARG4, pyrG*) using absolute quantification. The corresponding calculations of copy number of the three genes in AF293 by comparison of the Ct values confirmed that each gene was present in single copy. This outcome was also observed in other *A. fumigatus* isolates (Table 3) and confirmed that *FKS1* was suitable as a single copy control gene.

Detection of a multi copy gene in A. fumigatus

In order to accurately quantitate multi copy genes, it was necessary to demonstrate that *FKS1* could be used to quantitate a multi copy gene of known copy number. Furthermore, we were interested in knowing how discriminatory our strategy would be with regard to copy number accuracy. To make this determination we decided to use a multi copy gene that was present in low copy number and chose *arsC* as a target. Sequence analysis of *arsC* from the AF293 genome suggested that it was present in two copies, one copy on chromosome 1 and one copy on

chromosome 5. Careful inspection of the two sequences by DNA alignment revealed that a combination of primer position and restriction digestion would confirm the presence of two copies, after gel electrophoresis, based on the predicted sizes of digestion products of the PCR reaction (Fig. 1A). Figure 1B confirms that the predicted digestion patterns of the two *arsC* alleles matched what was observed in the gel after electrophoresis.

Quantitative real time PCR was next used to determine the copy number of *arsC* in AF293. The Ct values were determined for the *arsC* sequences and compared to *FKS1*, which was used as the single copy control. The output graph from the reaction shows an earlier Ct for *arsC* than for *FKS1* (Fig. 2), consistent with the greater copy number of *arsC*. Calculation of copy number of *arsC* for AF293 and for other isolates demonstrated that *arsC* is present in 2 copies (Table 4), which confirmed that our strategy could determine the copy number of multi copy sequences.

Determination of rDNA copy number

Once *FKS1* was established as a reliable single copy control, this sequence was used to determine the copy number of the rDNA genes in *A. fumigatus* by quantitating the copy number of the 18S ribosomal DNA subunit. Since the copy number of the rDNA genes of AF293 has been determined from the genome sequence, this isolate was used in a pilot Taqman assay in which *FKS1* (single copy) was used to calculate the copy number of the 18S rDNA subunit. According to the genome sequence, AF293 has 35 copies of the rDNA genes per genome (33). Figure 3 shows an example of the plots of *FKS1* vs. 18S rDNA and clearly demonstrates that there are more copies of the 18S rDNA gene than the *FKS1* gene. Calculation of the 18S rDNA copy numbers resulted in a value of 38 copies of 18S rDNA per genome in AF293, which is in

fairly close agreement with the genomic copy number (38 vs. 35 copies) for AF293 (33). The 18S rDNA copy numbers of the remaining isolates were then determined using *FKS1* as the reference gene. The data show a range of 38-91 copies, with an average of 54 copies per genome (Table 5). These results show that for our set of isolates, 18S rDNA copy number is isolate-specific and can vary substantially from strain to strain.

Stability of rDNA copy number

Since our results indicated that rDNA copy number could vary among strains of *A. fumigatus*, we investigated various environmental conditions to determine whether or not an affect on copy number could be observed. Factors that were investigated included morphology, growth temperature, culture age, antifungal exposure (itraconazole), and animal model organ site (lung vs kidney). The overall copy number mean was found to be 38.032 $\pm$ 0.13, which agrees with our initial copy number determination of AF293. However, Table 6 shows that some significant differences in copy number were observed among our growth conditions (morphology, growth temperature, culture age). In spite of these differences, variation in copy number among all conditions tested was less than 1 copy and would all have been 38 copies if numbers were rounded.

DISCUSSION

Timely diagnosis of IA is challenging due to the lack of specific clinical manifestations of infection. Unfortunately symptoms can be non-specific and include: fever, cough, dyspnea, chest pain, or apnea. Therefore, diagnosis can be dependent on the combination of a strong index of suspicion and radiologic findings, serologic assays, or when possible, culture and/or histologic

findings (24). For any of these methods, a quantitative estimate of fungal burden is difficult at best and can be expensive or time consuming. In fact, even under controlled experimental conditions of animal modeling, colony counts can be misleading as some studies have noted decreasing counts that are contradicted by other measurements in the same animal (35, 36). The ubiquitous nature of *A. fumigatus* in the environment and associated possibility of inhaling fungal elements that may or may not grow *in vivo*, but could be detected as CFU after lavage further complicates making an accurate assessment.

Through advances in instrumentation and reagent chemistry, PCR continues to find new applications in clinically relevant areas. In spite of not being widely employed as a routine clinical diagnostic tool for detecting IA, PCR is proving increasingly useful as an investigational tool for studying aspergillosis both *in vitro* and *in vivo*, and may ultimately find its way into the clinical laboratory as a routine diagnostic tool for IA. For *in vivo* applications of animal infections, qPCR is often used to make a determination of CFUs, which are frequently expressed as conidial equivalents in order to indicate one nucleus per conidium. While CFU is fairly accurate for fungi that grow in a yeast morphology, CFUs obtained by plate counts can be difficult to interpret for filamentous organisms due to the inability to distinguish a single hypha that forms one colony from the same fragmented hypha that yields multiple colonies. In fact, using CFU for measuring *A. fumigatus* fungal loads has been shown to yield equivocal results (6, 35). Therefore, alternative methods that don't require obtaining viable colony counts but provide some indication of fungal burden are potentially useful for quantifying fungal load of a given specimen. qRT-PCR is exceptionally well suited for this requirement. In fact, when all protocols are standardized, from infection model through tissue preparation, reproducible results can be obtained, even among interlaboratory studies (34).

The observations in this study add an important caveat for standardized procedures to now include working with the same *A. fumigatus* strain when qPCR quantitation using the rDNA genes is required. Our results have shown that using an 18S rDNA target requires prior knowledge of copy number in the strain of interest. In our small sample size, we found copy number to vary by as much as ~2.5x. Neither the upper limit nor the lower limit of 18S rDNA copy number is known, but it is almost certain to vary by a larger amount than we observed for our set of isolates. Consequently, 18S rDNA copy number cannot be assumed based on another value previously determined from an unrelated strain. This observation presently does not have direct clinical implications since qPCR is not routinely used to diagnose IA, and fungal burden is rarely part of any diagnosis since for at risk patients, a positive assay regardless of amount, is always cause for concern. However, accurate quantitation of *A. fumigatus* CFU has numerous applications, many of which have clinically relevant consequences. These include data generated from more than one strain, or testing unknown strains, in experiments measuring tissue burdens, *in vivo* drug susceptibility testing, environmental quantitation, tracking CFU during disease progression, or comparison of different methods for measuring fungal load (2, 17, 32, 35, 42). Similarly, direct quantitative comparisons of the same or different strains that utilize qPCR vs some other method, such as CFU or galactomannan, can be erroneous in the absence of an accurate rDNA copy number. Finally, model systems that may use the same assay but different strains and report results in CFU, such as animal survival studies, typically use absolute numbers and therefore, need to be calculated accurately if qPCR is part of the methodology. However, in spite of the variation between strains, our results suggest that within strain variation, at least in the case of AF293, is negligible. Therefore, in studies that utilize the same strain and involve quantitation, qPCR using the rDNA genes should yield consistent results. We could not identify

any condition that was able to cause the 18S subunit number to vary by more than 1 copy within AF293 in spite of investigating a number of stress conditions. However, we did identify some significant differences in our analyses. We suspect these differences may have been due to experimental error since qPCR accuracy requires precise technique. On the other hand, we know nothing about the mechanism by which copy number variation occurs and what, if any, phenotypic consequences are associated with changes in copy number within a strain. The fact that different strains of *A. fumigatus* have different rDNA copy numbers is evidence that variation occurs. Since our qPCR assay can only detect whole copies (a fraction of a copy would not yield a PCR product), the data could have arguably been rounded to the nearest whole copy. In this case, all copy numbers would round to 38, which matches the control AF293 number. However, since we cannot rule out copy number heterogeneity within a population, we chose not to round the data. Future studies of copy number should focus on whether changes are rapid, such as by an unequal recombination event that leads to large gains or losses of rDNA repeats, or gradual, which could result in small changes of a unit or two over longer periods of time. Understanding the mechanism may reveal whether or not the changes are responses to selection, or are random without clear phenotypic consequences.

In spite of the observed copy number variation within *A. fumigatus*, application of these results to other species of *Aspergillus* probably should not be done without empirical analysis. *Aspergillus* taxonomy can be complicated by the existence of sections, which may not be discriminated at the clinical level, but can be discriminated at the molecular level. For example, in the *Aspergillus* section *Fumigati*, *A. fumigatus* may not be discriminated from other members such as *A. lentulus* or *A. brevipes*. However, these species can be identified by sequencing select loci (ie., β-tubulin). Therefore, rDNA variation could possibly indicate a separate subspecies. In

359 our study, we confirmed that our strains were all *A. fumigatus* using β-tubulin sequencing (data
360 not shown), but since so little is known at the molecular level about these sub genera,
361 confirmational sequencing of additional loci may be required when trying to quantitate unknown
362 isolates.

363 Although we targeted the 18S rDNA subunit in this study, determination of copy number
364 should hold for targets that lie within the 28S subunit or between the two subunits (ITS1, ITS2,
365 5.8S) as well since the large and small ribosomal subunits, though multicopy and tandemly
366 arrayed, are colinear and transcribed as a single transcript along with the intervening ITS region
367 (21, 43). Therefore, based on what is known in model fungi, the copy number of the 18S and 28S
368 genes, as well as the intervening sequences, should be the same in the same strain of *A.*
369 *fumigatus*. The advantage of primer design in the more variable ITS1, ITS2 or even the D1/D2
370 region of the 28S subunit is that species specificity can be possible, subspecies issues as
371 described above not withstanding. If, on the other hand, the increased sensitivity of targeting the
372 multi copy rDNA genes is not needed, a suitable single copy gene (i.e., *FKS1, ARG4, pyrG*) can
373 be used with fairly high confidence that it will be invariant among unrelated strains and equal to
374 1. Finally for presence or absence outcomes, copy number variation is probably not a concern,
375 however, given that the ribosomal genes are usually targeted due to their increased sensitivity, if
376 investigators are quantitating cell numbers using these genes, the strain-specific variability of
377 rDNA copy number may be an important factor that affects the sensitivity of PCR assays for
378 quantifying *Aspergillus fumigatus.*
379

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REFERENCES

1. **Applied Biosystems, I.** 2001. Relative Quantitation of Gene Expression, User Bulletin #2.
2. **Baskova, L., C. Landlinger, S. Preuner, and T. Lion.** 2007. The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of *Aspergillus* and *Candida* species. J Med Microbiol **56:**1167-73.
3. **Beauvais, A., J. M. Bruneau, P. C. Mol, M. J. Buitrago, R. Legrand, and J. P. Latge.** 2001. Glucan synthase complex of *Aspergillus fumigatus*. J Bacteriol **183:**2273-9.
4. **Belkhiri, A., and G. R. Klassen.** 1996. Diverged 5s rRNA sequences adjacent to 5s rRNA genes in the rDNA of *Pythium pachycaule*. Curr Genet **29:**287-92.
5. **Boudewijns, M., P. E. Verweij, and W. J. Melchers.** 2006. Molecular diagnosis of invasive aspergillosis: the long and winding road. Future Microbiol **1:**283-93.
6. **Bowman, J. C., G. K. Abruzzo, J. W. Anderson, A. M. Flattery, C. J. Gill, V. B. Pikounis, D. M. Schmatz, P. A. Liberator, and C. M. Douglas.** 2001. Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. Antimicrob Agents Chemother **45:**3474-81.
7. **Brookman, J. L., and D. W. Denning.** 2000. Molecular genetics in *Aspergillus fumigatus*. Curr Opin Microbiol **3:**468-74.
8. **Challier, S., S. Boyer, E. Abachin, and P. Berche.** 2004. Development of a serum-based Taqman real-time PCR assay for diagnosis of invasive aspergillosis. J Clin Microbiol **42:**844-6.
9. **Chamilos, G., and D. P. Kontoyiannis.** 2006. Defining the diagnosis of invasive aspergillosis. Med Mycol **44 Suppl:**163-72.
10. **Costa, C., D. Vidaud, M. Olivi, E. Bart-Delabesse, M. Vidaud, and S. Bretagne.** 2001. Development of two real-time quantitative TaqMan PCR assays to detect circulating Aspergillus fumigatus DNA in serum. J Microbiol Methods **44:**263-9.
11. **D'Enfert, C., M. Diaquin, A. Delit, N. Wuscher, J. P. Debeaupuis, M. Huerre, and J. P. Latge.** 1996. Attenuated virulence of uridine-uracil auxotrophs of *Aspergillus fumigatus*. Infect Immun **64:**4401-5.
12. **Denning, D. W.** 1998. Invasive aspergillosis. Clin Infect Dis **26:**781-803; quiz 804-5.
13. **Denning, D. W.** 1996. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis **23:**608-15.
14. **Firon, A., A. Beauvais, J. P. Latge, E. Couve, M. C. Grosjean-Cournoyer, and C. D'Enfert.** 2002. Characterization of essential genes by parasexual genetics in the human fungal pathogen *Aspergillus fumigatus*: impact of genomic rearrangements associated with electroporation of DNA. Genetics **161:**1077-87.
15. **Fournier, P., C. Gaillardin, M. A. Persuy, J. Klootwijk, and H. van Heerikhuizen.** 1986. Heterogeneity in the ribosomal family of the yeast *Yarrowia lipolytica*: genomic organization and segregation studies. Gene **42:**273-82.
16. **Garber, R. C., B. G. Turgeon, E. U. Selker, and O. C. Yoder.** 1988. Organization of ribosomal RNA genes in the fungus *Cochliobolus heterostrophus*. Curr Genet **14:**573-82.
17. **Gomez-Lopez, A., M. T. Martin-Gomez, P. Martin-Davila, P. Lopez-Onrubia, J. Gavalda, J. Fortun, A. Pahissa, J. L. Rodriguez-Tudela, and M. Cuenca-Estrella.** 2006. Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2

methodologies in experimental pulmonary aspergillosis. Diagn Microbiol Infect Dis **56:**387-93.

18. **Gonzalez, E., H. Kulkarni, H. Bolivar, A. Mangano, R. Sanchez, G. Catano, R. J. Nibbs, B. I. Freedman, M. P. Quinones, M. J. Bamshad, K. K. Murthy, B. H. Rovin, W. Bradley, R. A. Clark, S. A. Anderson, J. O'Connell R, B. K. Agan, S. S. Ahuja, R. Bologna, L. Sen, M. J. Dolan, and S. K. Ahuja.** 2005. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science **307:**1434-40.

19. **Hohl, T. M., and M. Feldmesser.** 2007. *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryot Cell **6:**1953-63.

20. **Howlett, B. J., B. D. Rolls, and A. J. Cozijnsen.** 1997. Organisation of ribosomal DNA in the ascomycete *Leptosphaeria maculans*. Microbiol Res **152:**261-7.

21. **Iwen, P. C., S. H. Hinrichs, and M. E. Rupp.** 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol **40:**87-109.

22. **Jin, J., Y. K. Lee, and B. L. Wickes.** 2004. Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol **42:**4293-6.

23. **Khan, Z. U., S. Ahmad, and A. M. Theyyathel.** 2008. Detection of *Aspergillus fumigatus*-specific DNA, (1-3)-beta-d-glucan and galactomannan in serum and bronchoalveolar lavage specimens of experimentally infected rats. Mycoses **51:**129-35.

24. **Kontoyiannis, D. P., and G. P. Bodey.** 2002. Invasive aspergillosis in 2002: an update. Eur J Clin Microbiol Infect Dis **21:**161-72.

25. **Latge, J. P.** 1999. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev **12:**310-50.

26. **Loeffler, J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele.** 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J Clin Microbiol **38:**586-90.

27. **Loeffler, J., K. Kloepfer, H. Hebart, L. Najvar, J. R. Graybill, W. R. Kirkpatrick, T. F. Patterson, K. Dietz, R. Bialek, and H. Einsele.** 2002. Polymerase chain reaction detection of *Aspergillus* DNA in experimental models of invasive aspergillosis. J Infect Dis **185:**1203-6.

28. **Lopez-Ribot, J. L., R. K. McAtee, W. R. Kirkpatrick, S. Perea, and T. F. Patterson.** 2000. Comparison of DNA-based typing methods to assess genetic diversity and relatedness among Candida albicans clinical isolates. Rev Iberoam Micol **17:**49-54.

29. **Maleszka, R., and G. D. Clark-Walker.** 1990. Magnification of the rDNA cluster in *Kluyveromyces lactis*. Mol Gen Genet **223:**342-4.

30. **Maleszka, R., and G. D. Clark-Walker.** 1993. Yeasts have a four-fold variation in ribosomal DNA copy number. Yeast **9:**53-8.

31. **Marr, K. A., R. A. Carter, M. Boeckh, P. Martin, and L. Corey.** 2002. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood **100:**4358-66.

32. **McDevitt, J. J., P. S. Lees, W. G. Merz, and K. J. Schwab.** 2004. Development of a method to detect and quantify *Aspergillus fumigatus* conidia by quantitative PCR for environmental air samples. Mycopathologia **158:**325-35.

33. **Nierman, W. C., A. Pain, M. J. Anderson, J. R. Wortman, H. S. Kim, J. Arroyo, M. Berriman, K. Abe, D. B. Archer, C. Bermejo, J. Bennett, P. Bowyer, D. Chen, M. Collins, R. Coulsen, R. Davies, P. S. Dyer, M. Farman, N. Fedorova, N. Fedorova, T. V. Feldblyum, R. Fischer, N. Fosker, A. Fraser, J. L. Garcia, M. J. Garcia, A. Goble, G. H. Goldman, K. Gomi, S. Griffith-Jones, R. Gwilliam, B. Haas, H. Haas, D. Harris, H. Horiuchi, J. Huang, S. Humphray, J. Jimenez, N. Keller, H. Khouri, K. Kitamoto, T. Kobayashi, S. Konzack, R. Kulkarni, T. Kumagai, A. Lafon, J. P. Latge, W. Li, A. Lord, C. Lu, W. H. Majoros, G. S. May, B. L. Miller, Y. Mohamoud, M. Molina, M. Monod, I. Mouyna, S. Mulligan, L. Murphy, S. O'Neil, I. Paulsen, M. A. Penalva, M. Pertea, C. Price, B. L. Pritchard, M. A. Quail, E. Rabbinowitsch, N. Rawlins, M. A. Rajandream, U. Reichard, H. Renauld, G. D. Robson, S. Rodriguez de Cordoba, J. M. Rodriguez-Pena, C. M. Ronning, S. Rutter, S. L. Salzberg, M. Sanchez, J. C. Sanchez-Ferrero, D. Saunders, K. Seeger, R. Squares, S. Squares, M. Takeuchi, F. Tekaia, G. Turner, C. R. Vazquez de Aldana, J. Weidman, O. White, J. Woodward, J. H. Yu, C. Fraser, J. E. Galagan, K. Asai, M. Machida, N. Hall, B. Barrell, and D. W. Denning.** 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature **438:**1151-6.

34. **Sheppard, D. C., J. R. Graybill, L. K. Najvar, L. Y. Chiang, T. Doedt, W. R. Kirkpatrick, R. Bocanegra, A. C. Vallor, T. F. Patterson, and S. G. Filler.** 2006. Standardization of an experimental murine model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother **50:**3501-3.

35. **Sheppard, D. C., K. A. Marr, D. N. Fredricks, L. Y. Chiang, T. Doedt, and S. G. Filler.** 2006. Comparison of three methodologies for the determination of pulmonary fungal burden in experimental murine aspergillosis. Clin Microbiol Infect **12:**376-80.

36. **Singh, G., J. Imai, K. V. Clemons, and D. A. Stevens.** 2005. Efficacy of caspofungin against central nervous system *Aspergillus fumigatus* infection in mice determined by TaqMan PCR and CFU methods. Antimicrob Agents Chemother **49:**1369-76.

37. **Spiess, B., D. Buchheidt, C. Baust, H. Skladny, W. Seifarth, U. Zeilfelder, C. Leib-Mosch, H. Morz, and R. Hehlmann.** 2003. Development of a LightCycler PCR assay for detection and quantification of *Aspergillus fumigatus* DNA in clinical samples from neutropenic patients. J Clin Microbiol **41:**1811-8.

38. **Standards, National Committee for Clinical Laboratory.** 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.

39. **Townson, J. R., L. F. Barcellos, and R. J. Nibbs.** 2002. Gene copy number regulates the production of the human chemokine CCL3-L1. Eur J Immunol **32:**3016-26.

40. **Tsai, H. F., Y. C. Chang, R. G. Washburn, M. H. Wheeler, and K. J. Kwon-Chung.** 1998. The developmentally regulated alb1 gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J Bacteriol **180:**3031-8.

41. **van Burik, J. A., R. W. Schreckhise, T. C. White, R. A. Bowden, and D. Myerson.** 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med Mycol **36:**299-303.

42. **van Vianen, W., S. de Marie, M. T. ten Kate, R. A. Mathot, and I. A. Bakker-Woudenberg.** 2006. Caspofungin: antifungal activity in vitro, pharmacokinetics, and effects on fungal load and animal survival in neutropenic rats with invasive pulmonary aspergillosis. J Antimicrob Chemother **57:**732-40.

43. **Warner, J. R.** 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol Rev **53:**256-71.
44. **Weidner, G., C. d'Enfert, A. Koch, P. C. Mol, and A. A. Brakhage.** 1998. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. Curr Genet **33:**378-85.

FIGURE LEGENDS

FIGURE 1. Confirmation of *arsC* copy number in AF293. A) Priming sites for the two *arsC* alleles. The Chr1 *arsC* allele is located on chromosome 1 while the Chr5 *arsC* allele is located on chromosome 5. Primers are indicated by black arrows, PCR product is indicated by the line connecting the primers. The three *Nco* I sites (N) (1 located within and 2 flanking the *arsC* genes) with location in bp, are indicated within parentheses. Stippled boxes are the *arsC* orfs. The predicted sizes of the fragments after *Nco* I digestion are indicated below each orf. B) *Nco* I digestion of *arsC* PCR products. Lane 1, uncut Chr1 *arsC* PCR product, lane 2, *Nco* I digest of Chr1 *arsC*, lane 3, mixture of both *Nco* I digestions, lane 4, *Nco* I digestion of Chr5 *arsC* PCR product, lane 5, uncut Chr5 *arsC* PCR product. Sizes are in base pairs. L = ladder. Ladder sizes are on the right of the gel, fragment sizes are on the left.

FIGURE 2. Amplification plot of AF293 *arsC* vs *FKS1* taqman assays. Taqman assays were performed using an *arsC* primer probe combination and *FKS1* primer probe combination. The graph represents a sample plot from duplicate reactions run on aliquots of the same DNA sample. Amplification of the *arsC* gene is denoted by circles. Amplification of the *FKS1* gene is denoted by squares. The Ct value of the *arsC* line is approximately 21.1 (downward arrow) and the Ct value of *FKS1* is approximately 22.1 (upward arrow).

FIGURE 3. Amplification plot of 18S rDNA vs *FKS1*. An example of copy number determination of 18S rDNA using *FKS1* as a single copy control. The figure is an amplification plot of a Taqman assay done using the 18S rDNA primer-probe combination and *FKS1* primer probe combination. Template DNA was taken from the same DNA sample prepared from AF293 and run in duplicate. Note the earlier Ct value of 18S rDNA (circles), which is approximately 18.0 (downward arrow) vs. the *FKS1* Ct value (squares), which is approximately 23.4 (upward arrow). The lower Ct value for 18S rDNA reflects the greater copy number of the target since the fluorescence crosses the threshold at a much lower cycle number.

Table 1. Strains used in this study.

Strain	Source	Contributor/Strain alias/Reference
AF293	Clinical	R. Aramayo
WSA[1]-172	Clinical	M. Rinaldi (#98-407)
WSA-270	Clinical	ATCC#64746
WSA-271	Clinical	ATCC#14110
WSA-419	Clinical	K.J. Kwon-Chung (#B-5233) (40)
WSA-445	Clinical	T. Patterson (# MTFP0009)
WSA-446	Clinical	M. Rinaldi (#99-1900)
WSA-621	Clinical	B. Lutz (#1)

[1]WSA isolates are isolates from the Wickes laboratory culture collection

Table 2. PCR primer and probe sequences.

Primer/Probe	Sequence	Ref.
18S rDNA.F 18S rDNA.R 18S rDNA probe[1]	5'-GGCCCTTAAATAGCCCGGT-3' 5'-TGAGCCGATAGTCCCCCTAA-3' 6-FAM-AGCCAGCGGCCCGCAAATG-MGBNFQ	(10)
AFKS.F[2] AFKS.R AFKS probe AFKS probe	5'-GCCTGGTAGTGAAGCTGAGCGT-3 5'-CGGTGAATGTAGGCATGTTGTCC-3' 6- FAM-TCACTCTCTACCCCCATGCCCGAGCC-MGBNFQ 6- VIC-TCACTCTCTACCCCCATGCCCGAGCC-MGBNFQ	(6)
ARG4.F ARG4.R ARG4 probe	5'-CAGCCCCGGGAAACTCA-3' 5'-TCCGCTCCCTTGACAGCTT-3' 6- FAM-CCAGACCAATGTTCCTGAG-MGBNFQ	This study
pyrG.F pyrG.R pyrG probe	5'-TGGCCCAGACCGCATCT-3' 5'-CAACAGTCCTCTCTCAGGACCAT-3' 6- VIC-CGCAAGACTTCCC-MGBNFQ	This study
arsC.F arsC.R arsC probe	5'-GCCGCTGGGTTCCTTACTC-3' 5'-CAGCGGAGCGAACCTCAATA-3' 6- FAM-CCTCGCAGGTGATG-MGBNFQ	This study
Chr1arsC.F[3] Chr1arsC.R Chr5arsC.F Chr5arsC.R	5'–GACCTCGACACCCTAAGAAGC-3' 5'–TCAAATGATGAGAGGCCAGA-3' 5'–TCCTCCATCTTCATTCCCTTA-3' 5'–GAGCTGGAACCTCAGCGTAG-3'	This study

[1]MGB probe dyes are incorporated into the primer sequences, i.e., 6-FAM-AGCCAGCGGCCCGCAAATG-MGBNFQ is an MGB probe labeled with FAM.
[2]AFKS primers and probes were used for detection of the *FKS1* gene. *ARG4*, *pyrG* and *arsC* primers and probes were used for detection of *ARG4*, *pyrG* and *arsC* genes.
[3]Primers designated Chr1arsC or Chr5arsC are for routine PCR amplification of the two *arsC* alleles from chromosome 1 or 5.

Table 3. Confirmation of copy number of predicted single copy genes.

Strain	*FKS1* Avg Ct[α]	*FKS1* copy#	*pyrG* Avg Ct	*pyrG* copy#	*ARG4* Avg Ct	*ARG4* copy#
AF293	19.16 +/-.012[b]	1	19.33 +/-.005	1.13	19.06 +/- .008	1.07
WSA-172	21.39 +/-.012	1	21.34 +/-.001	0.97	21.23 +/-.007	1.11
WSA-445	22.26 +/-.049	1	22.55 +/-.003	1.22	22.31 +/-.004	0.97
WSA-621	24.34 +/-.014	1	23.52 +/-.004	1.29	24.54 +/- .007	1.10
WSA-419	21.40 +/-.050	1	21.82 +/-.003	1.34	21.40 +/-.005	1.00

[a]Threshold cycle
[b] Mean +/- standard deviations.
N= 3 samples, run in duplicate. Copy # of test gene (*pyrG* or *ARG4*) = $2^{-\Delta Ct}$

Table 4. Determination of *arsC* copy number by qRT-PCR, *FKS1* vs *arsC*, all isolates.

Strains	Avg C_t *FKS1*[a]	*FKS1* Copy#	Avg C_t *arsC*	*arsC* copy#[b]
AF293	22.1 +/-.107	1	21.1 +/-.007	2 (2.00)
WSA-172	19.8 +/-.010	1	18.7 +/-.004	2 (2.14)
WSA-446	22.0 +/-.052	1	21.1 +/-.003	2 (1.89)
WSA-445	19.8 +/-.014	1	18.7 +/-.005	2 (2.16)
WSA-271	22.1 +/-.025	1	21.0 +/-.002	2 (2.07)
WSA-270	19.2 +/-.042	1	18.2 +/-.002	2 (2.00)
WSA-621	19.9 +/-.060	1	18.8 +/-.003	2 (2.01)
WSA-419	23.1 +/-.014	1	21.9 +/-.007	2 (2.42)

[a] [a]Mean +/- standard deviations.

[b] *arsC* copy # was determined using the formula $2^{-\Delta C_t}$. Results shown in parentheses, which were then rounded to whole numbers. N= 3 samples run in duplicate.

Table 5. *A. fumigatus* 18s rDNA copy number determinations.

Strain	18s rDNA copies [a]
AF293	38 +/- 0.01
WSA-172	46 +/- 0.03
WSA-446	47 +/- 0.01
WSA-445	49 +/- 0.06
WSA-271	49 +/- 0.05
WSA-270	53 +/- 0.01
WSA-621	70 +/- 0.03
WSA-419	91 +/- 0.03

[a]Mean +/- standard deviations.
N= 3 samples run in duplicate.

Table 6. *A. fumigatus* 18S rDNA copy number stability.

Condition	Subgroup	18s rDNA Copies[a]	P-value
Morphology	Conidia	38.02 ± 0.011	
	Hyphae	38.11 ± 0.01	0.03
Temperature	30°C Growth	37.84 ± 0.044	
	45°C Growth	38.03 ± 0.015	0.03
Culture Age	3d Growth	38.041 ± 0.024	
	5d Growth	37.906 ± 0.059	
	10d Growth	38.321 ± 0.019	0.004
	25d Growth	38.061 ± 0.017	
Itraconazole	0.00 µg/ml	37.984 ± 0.049	
	0.03 µg/ml	38.024 ± 0.015	
	0.06 µg/ml	38.039 ± 0.022	
	0.125 µg/ml	38.033 ± 0.012	0.17
	0.25 µg/ml	38.001 ± 0.055	
	0.5 µg/ml	38.046 ± 0.009	
	1.0 µg/ml	38.039 ± 0.019	
	2.0 µg/ml	38.034 ± 0.038	
Mouse	Lung	38.056 ± 0.038	
	Kidney	38.136 ± 0.008	0.13
Guinea Pig	Lung	38.231 ± 0.008	
	Kidney	37.688 ± 0.059	0.13

[a]Mean +/- standard deviations.